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Preparation and Anti-Insulin Activity of Lipoprotein Fractions from Rat Serum.* (24813)

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Lipoprotein fractions from diabetic rat serum have been reported to inhibit glucose utilization by muscle(1) and by hexokinase in cell-free systems(2). The role of lipoproteins as regulators of enzyme activity is now under examination(3,4,5,6). These studies have been handicapped by the fact that published fractionation and test procedures require diabetic serum as starting material and are not sufficiently explicit to yield products which are always inhibitory. The present paper describes methods for preparation of uniformly inhibitory fractions; they are applicable not only to diabetic, but also to normal rat serum. Ability of inhibitory fractions to block insulin action on muscle has been estimated.

Methods and results. Measurements of glucose uptake. Fractions have been tested for ability to inhibit glucose uptake by diaphragms of normal rats, especially in presence of small concentrations of insulin. Strain, sex, weight, and feeding schedule of rats, environmental temperature, removal of diaphragms and their handling and incubation must be scrupulously standardized. Experimental de-

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tails essential for reproducibility follow. Diaphragm donors were male Sprague-Dawley rats, received as groups of 24 to 36 born on same day and kept in laboratory 7-14 days at 25°C, food available at all times. Rats used on given day were fasted 24 hours; individuals within weight range of 95-105 g were selected; animals below 90 g yielded unsatisfactory results. Weight range was constant. Insulin solutions were prepared fresh daily by dilution of Iletin (Insulin, Lilly), 80 units/ml, with Krebs-bicarbonate solution; dilutions were made to minimize loss of insulin on glass. To prevent chance insulin pollution all glass vessels were washed in chromic acid; electrodes of pH meter were washed with 1 N NaOH and then with 1 N HCl (in 50% ethanol) before each preparative run. All plastic tubes were new; all syringes and needles were soaked 24 hours or more in alkaline detergent after use. *Rat sera.* In extending original work(1) rats with severe alloxan diabetes, prepared as before (5), were used. Blood was obtained from heart, allowed to clot 5 minutes at 25°C, then centrifuged (4500 g) 15 minutes at 5°. All subsequent operations were carried out at 5° or below until incubation of diaphragms was begun. Samples representing desired degree of diabetes (as judged by serum sugar(8) level) and having desired lipide(5,9) content (see below) were pooled for fractionation. Fractions to be tested were dissolved in protein-containing medium to replace normal milieu of fraction, at first in diabetic rat serum from which inhibitory activity had been removed by repeated freezing and thawing(5, 10), then, for simplicity, in 6% solutions of Armour bovine fraction V (crude albumin) in Krebs-bicarbonate buffer. Several lots of fraction V were tried; lot No. M-12011 was satisfactory without further treatment; lot No. R-14005 was satisfactory when 7.3% solution in Krebs salt solution (without bicarbonate or CO_2) was dialysed 2 hours at 5° against the same salt solution, protein concentration being adjusted to 6% and bicarbonate concentration to that of Krebs solution before incubation; glucose concentration was 600 mg % to facilitate comparison of inhibitory effects of diabetic sera and fractions therefrom. Commercial samples of crystalline bovine albumin inhibited glucose uptake of diaphragms. Each sample of pooled serum and each precipitated fraction (in volume of Krebs salt solution without bicarbonate equal to that of parent serum) was subjected to zone electrophoresis on paper(5); results are summarized in terms of 2 fractions: albumin plus α-globulin (Aα) and β-globulin plus γ-globulin ($\beta\gamma$). Other analyses were: total protein (11), total and free cholesterol(12), phospholipide(13), total(9) and protein-bound(5) lipids. The protein-bound lipids, as here defined, averaged 83% of total lipide. *Precipitation of inhibitory fractions.* Initial reports on separation of inhibitor from diabetic rat serum stated that activity was obtained in Cohn combined fraction I, II, III(1,2). Here, the first attempts to repeat and extend these observations were unsuccessful. Diabetic rat sera were subjected to Oncley-Cohn procedure for precipitation of combined fraction I, II, III from normal human plasma(14,15). Sixteen samples of the fraction were prepared and dissolved in frozen-thawed(5) diabetic serum for test of inhibitory activity; 8 were inhibitory and 8 were inactive, average inhibition when present being 1.2 mg/g diaphragm/hour. Inhibitory activity tended to be associated with a high ratio of β- to α-lipoprotein in the fraction, and vice versa. The clue to preparation of uniformly inhibitory fractions was found in 2 modifications: preparation of fractions with all practicable speed, and use of slightly lower alcohol concentration and higher ionic strength than employed for human plasma(14,15). Electrophoretic patterns for fractions prepared from rat serum by Oncley-Cohn, and by present method are shown in Fig. 1. Procedure selected for precipitation and test of inhibitory fractions from rat sera was as follows. 1. Precipitating reagent was made up fresh daily and cooled to –5°. It contained 10 ml 95% ethanol, .15 ml .8 M sodium acetate-acetic acid buffer (pH 4), 1 ml .06 M NaCl, .08 ml 1 M acetic acid; the volume was made to 50 ml with water. 2. Samples of rat serum containing less than 700 mg % protein-bound lipide were pooled and

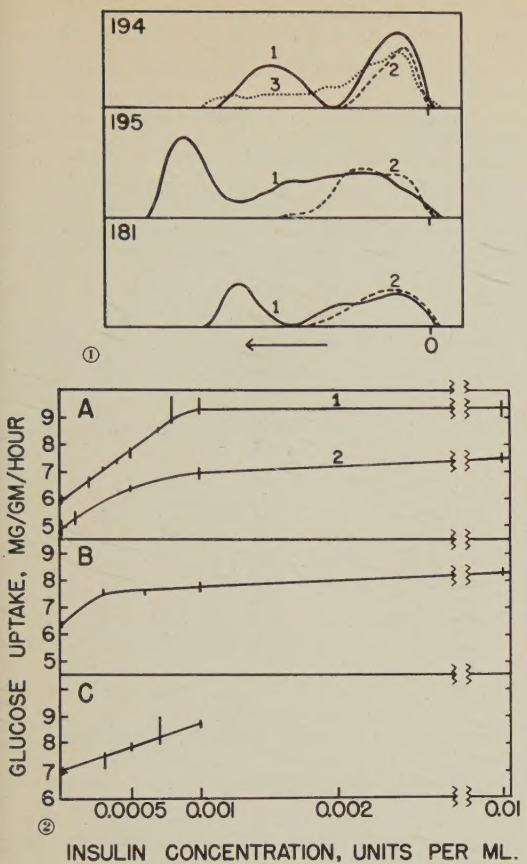

FIG. 1. Zone electrophoresis patterns for sera and inhibitory fractions after staining for lipides (5). The origin is at 0. No. 194, 195, 181 refer to same experiments as in Tables I-II. Exp. 194, fasting normal rats: 1, serum; 2, fraction prepared by present modified Oncley-Cohn method; 3, fraction from same serum by original Oncley-Cohn method; 0.02 ml serum or solution of fraction was used for each run. Exp. 195, fasting normal rats: 1, serum; 2, fraction by present method; 0.03 ml serum or fraction. Exp. 181, fasting diabetic rats: 1, serum; 2, fraction by present method; 0.013 ml serum or fraction. For other details see text.

FIG. 2. Relation of glucose uptake of diaphragms from normal fasting rats to insulin concentration in incubation medium. A, in Krebs-bicarbonate medium; B, in a solution of 6% Armour bovine fraction V in Krebs-bicarbonate; C, in diabetic rat serum which had been repeatedly frozen and thawed. Glucose concentration was 600 mg % except in A, curve 2, where it was 300 mg %. Length of each vertical line represents 2 standard errors, one on each side of mean. For other details, see text.

chilled to 0°. Samples with obvious hemolysis were excluded. The fractionation *must* be begun within less than 1 hour after blood is drawn. 3. Two-tenths ml of pooled serum was mixed with .8 ml of precipitation reagent, 4 ml of .15 M NaCl were added, pH read at 25°; if pH was not 6.2 ± 0.2, 1 M acetic acid was added to the reagent, pH test was repeated; this was continued until a reagent giving proper pH was obtained. The maximum amount of 1 M acetic acid required/50 ml was about .16 ml for diabetic serum and .23 ml for normal serum. These preliminary tests must be carried out before precipitation of main pooled serum sample is begun, and with greatest expedition, *i.e.*, in 10-15 minutes or less. 4. Four volumes of adjusted precipitation reagent were added dropwise with stirring at −5° to main pooled serum samples during 10 minutes. The mixture was stirred a further 5 minutes, then centrifuged promptly at 8000 g for 5 minutes at −5°. 5. The protein precipitate was redissolved in a solution of bovine fraction V to give a volume equivalent to that of serum from which it was prepared. By trial it was found that the precipitate contributed about 10% of final volume, and 20-25% of protein of the solution; volume and concentration of fraction V solution were adjusted accordingly to make total protein concentration 6%. 6. One ml of solution of the protein fraction to be tested was transferred to each incubation beaker at 5°. Diaphragms were then removed from donor rats, pre-soaked(7) 15 minutes at 5°, and transferred to incubation beakers containing the solution of fraction V, or fraction V plus fraction under test; each incubation beaker received 2 halves of diaphragm from one rat. All samples were then incubated with shaking at 37° for 1 hour, removed, chilled and analyzed for glucose disappearance(5). Three to 6 aliquots of medium containing the fraction were incubated in this manner concurrently with the same number of aliquots of the medium without fraction.

Inhibitory activity of fractions from diabetic rat sera (Table I) was not correlated with protein-bound lipide or glucose levels of sera. When this was settled, fractions were prepared from serum of fasting normal rats; these were just as inhibitory as fractions from diabetic rat sera. No inactive (non-inhibitory) fractions have been obtained with above

TABLE I. Inhibitory Activity of Fractions from Diabetic or Normal Rat Sera of Varying Lipide and Glucose Content. Experiments have same numbers as in Table II and Fig. 1; arranged in the order as performed. For details, see *Methods*.

Exp. No.	Glucose uptake of diaphragms, mg/g/hr ± S.E.* In albumin + 0.0003 unit insulin/ml	+ fraction	Inhibition	Protein-bound lipide of pooled serum sample, mg % Avg	Range of individual sera	Glucose of pooled serum sample, mg % Avg	Range of individual sera	pH at which fraction was made
			Fractions from diabetic rat serum					
173	7.3 ± .45	6.3 ± .17	1.0	240	230– 245	488	308– 625	5.9
176	7.9 ± .07	6.5 ± .22	1.4	728	545–1100	755	542–1122	6.0
178	8.1 ± .32	7.0 ± .12	1.1	600	275–1060	1100	885–1325	6.1
180	6.6 ± .17	5.4 ± .04	1.2	356	302– 430	1010	659–1512	6.0
181	7.9 ± .28	6.4 ± .23	1.5	285	278– 288	1015	680–1435	5.9
183	7.7 ± .07	6.8 ± .23	.9	277	255– 345	670	516– 902	6.2
184	8.0 ± .09	6.2 ± .15	1.8	344	240– 550	990	510–1545	6.0
Means	7.64 ± .13†	6.37 ± .12†	1.27					
			Fractions from normal rat serum					
190	7.5 ± .31	6.1 ± .14	1.4	260	250–266	116	114–124	6.0
191	7.6 ± .27	6.5 ± .11	1.1	252	244–261	113	97–156	6.1
193	7.3 ± .14	6.4 ± .54	.9	252	248–258	109	76–140	6.1
194	6.7 ± .12	5.8 ± .37	.9	244	237–256	104	81–140	6.2
195	7.8 ± .57	6.8 ± .27	1.0	258	222–278	99	74–124	6.2
198	7.8 ± .36	6.5 ± .36	1.3	258	244–273	115	87–130	6.1
199‡	7.8 ± .36	6.5 ± .06	1.3	255	244–278	142	115–206	6.1
Means	7.50 ± .16§	6.37 ± .12§	1.13					

* Mean ± stand. error for all control samples in albumin alone was 6.4 ± 0.04.

† P value for the difference, as estimated by Fisher procedure for small numbers, is less than 0.001.

‡ Serum donors in this experiment had food available until time of bleeding.

§ P value for difference is less than 0.001.

procedure under conditions shown. However, non-inhibitory fractions could be obtained if sera were kept at room temperature for 1 or more hours before fractionation, if the fraction was exposed to ethanol solution for 30 minutes or more, or if individual sera with protein-bound lipide values above 1100 mg % were included in serum pool. These experiments show that inhibition is not an artifact arising from the reagents. Inhibitory fractions could be stored at −5° as precipitated protein cake (before re-solution in crude albumin medium) for 4 days without loss of activity.

Inhibitory activity of β-fraction was enhanced by i.p. injection into serum donor of 2 mg growth hormone, half at 24 hours, half at 3 hours prior to sacrifice. In 2 experiments (12 diaphragms) average inhibition of glucose uptake was 3.6 mg/g/hour as compared with 1.1 for uninjected controls. Adrenocorticotrophic and thyrotrophic hormones at same dose had no effect.† It was previously shown that inhibitory activity of diabetic *serum* was related to growth hormone(6,10).

Lipides of inhibitory fractions. Inhibitory activity was clearly not dependent on presence of albumin or α-globulin electrophoretic components, as inhibitory fractions free of Aα-proteins or lipides could be prepared from either diabetic or normal rat serum (Fig. 1, Table II). In fact, inhibitory β-fractions could be obtained from normal serum which was not in itself inhibitory. The Aα-fractions thus tended to prevent inhibition by the β-fraction. Protein and lipides of inhibitory fractions of Table I were further analyzed. For solutions of βγ-fraction in a volume of solution equal to that of original serum, mean values were (g/100 ml), for fractions from diabetic serum: protein, 1.34; total lipide, .332; cholesterol, .016 free, .43 esters; phospholipides, .024. For fractions from normal

† Growth hormone (Lot no. M208), ACTH (Lot no. 212-10) and TSH (Lot no. 6) were kindly supplied by Dr. Irby Bunding, Armour Labs.

TABLE II. Distribution of Protein-Bound Lipide between A_α- and $\beta\gamma$-Electrophoretic Components of Sera and of Inhibitory Fractions. Values expressed as g of protein-bound lipide/100 ml of serum or solution of fraction in Krebs-bicarbonate medium (see *Methods*). Experiments have same numbers, arranged in the same order, as in Table I.

Exp. No.	Pooled serum A_α	Pooled serum $\beta\gamma$	Inhibitory fraction A_α	Inhibitory fraction $\beta\gamma$
	Samples from diabetic rats			
173	.051	.193	.012	.019
176	.194	.478	.102	.438
178	.060	.486	.012	.478
180	.073	.284	.00	.185
181	.080	.205	.00	.127
183	.177	.128	.070	.066
184	.064	.325	.00	.316
Means	.100	.299	.019	.257
	Samples from normal rats			
190	.070	.195	.00	.133
191	.109	.177	.00	.063
193	.118	.174	.011	.099
194	.120	.175	.00	.100
195	.075	.202	.00	.131
198	.121	.144	.00	.163
199	.107	.195	.00	.187
Means	.103	.181	.002	.125

serum they were: protein, 1.31; total lipide, .150; cholesterol, .017 free, .034 esters; phospholipides, .030. The corresponding lipide values for normal rat serum were: total, .343; cholesterol, .032 free, .074 esters; phospholipide, .099. The fact that non-inhibitory fractions with electrophoretic patterns, not significantly different from those for inhibitory fractions, can be obtained from stored serum suggests that inhibition is associated with some component of β-lipoprotein which is quantitatively minor or with some particular molecular arrangement not revealed by methods hitherto applied.

Interaction between insulin and inhibitory fractions. Serum fractions inhibitory toward glucose uptake negated stimulatory effect of measurable amount of insulin. Dose-response curves (Fig. 2) relating concentration of insulin to glucose uptake of normal diaphragm were constructed for 3 incubation media: (a) Krebs-bicarbonate buffer, (b) 6% solution of Armour's bovine fraction V in Krebs-bicarbonate buffer, and (c) diabetic rat serum in which inhibitory activity had been destroyed by repeated freezing and thawing(5,10). Then fractions to be evaluated were dissolved in one of these media containing appropriate low concentration of insulin; glucose uptake of normal diaphragms in this solution was measured, and inhibition relative to concurrent control without the fraction was obtained. The amount of insulin corresponding to this reduction in rate of glucose uptake was estimated from dose-response curve; accuracy of the estimate is limited by flatness of curve. In the 6% fraction V solution, average inhibition induced by the fraction from 1 ml of diabetic rat serum was 1.3 mg/g muscle/hr (Table I). Fractions from normal rat serum were not significantly less inhibitory. From B (Fig. 2) this inhibition corresponded to negation 0.0003 unit of insulin/ml. In preliminary tests mentioned above, in which frozen-thawed diabetic serum was used as solvent for the inhibitory fraction the average inhibition was 1.2 mg/hr. From C (Fig. 2) this also corresponded to negation of about .0003-.0004 unit insulin/ml.

An independent estimate of amount of insulin blocked by the inhibitor of diabetic rat serum can be obtained from the difference in glucose uptake of normal diaphragms in diabetic rat serum, and in diabetic rat serum after inactivation of inhibitor by storage or freezing and thawing. In 2 series(5) values of the difference were 1.3 and 1.6, average of 1.45 mg/g/hour. From C (Fig. 2) this would correspond to freeing of .0007 unit of insulin/ml when total inhibitor in whole serum was inactivated. Since both estimates are likely to be minimal rather than maximal, it is provisionally concluded that the inhibitor in 1 ml of fasting rat serum can prevent stimulatory effect on muscle of at least .0004 to .001 unit of insulin.

The relation of these inhibitory lipoprotein fractions to the physiological action of insulin, and to other substances which interact with insulin has been discussed(6,16).

Summary. 1. Procedures for routine preparation from rat serum of fractions uniformly inhibitory to glucose uptake of rat diaphragm have been developed; it was possible to obtain such fractions not only from diabetic rat serum, as previously reported, but also from normal rat serum. Inhibitory activity of frac-

tions from normal serum was enhanced by prior treatment of serum donor with pituitary growth hormone; adrenocorticotrophic hormone or thyrotrophic hormone had no such effect. 2. Inhibitory fractions had gross properties of β-lipoproteins and could be prepared free of lipides having electrophoretic mobility of albumin or α-globulins. Average ratio of total lipide to protein was .25 for fractions from diabetic rat serum and .11 for fractions from normal rat serum; phosphatide/total lipide ratio was lower in such fractions than in parent sera. Relation of inhibitory activity to lipide content requires further clarification, as inhibitory activity may be lost by delay during preparation of fractions without significant change of lipide content or electrophoretic behavior of fractions. 3. Inhibitory fractions interfered with stimulatory action of insulin on diaphragm muscle. The fraction from 1 ml of diabetic or normal rat serum nullified the stimulatory effect of at least .0004-.001 unit of insulin.

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Net Synthesis of Heme from Protoporphyrin and Iron by Extracts of Duck Erythrocytes.* (24814)

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Hemin obtained from incubated mixtures containing labeled ionic iron, protoporphyrin, and preparations of avian erythrocytes was found to contain appreciable labeled iron; when the porphyrin or cell preparation was omitted from mixtures, or mixtures were not incubated, little labeled iron was recovered as hemin(1-4). From these findings and others, in particular the heat-lability of the cell preparations, it was inferred that in avian erythrocytes protoporphyrin and ionic iron are the immediate precursors of protoheme and that some component(s) of the erythrocytes, participates in heme formation. The inferences are reasonable; however, the evidence, at least for formation of heme from the presumed precursors, would be supplemented by establishing net synthesis of heme from protoporphyrin and iron in the presence of red-cell preparations. In particular, net synthesis would rule out the possibility that the recovery of labeled iron as hemin indicated above arose from artifact. The present report provides evidence

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for net synthesis of heme from iron and protoporphyrin in the presence of soluble material from duck erythrocytes.

Methods. Extracts of red cells were made as follows(2): at 1-4° cells were mixed with 2 volumes of water and let stand 30 minutes. One part of 1.72 *M* potassium chloride was added per 100 parts of added water. The mixture was centrifuged at about 3 x 10^4 x g for 30 minutes. The sediment was washed twice in the centrifuge with 0.156 *M* potassium chloride and suspended in an equal volume of the dilute chloride. Tween 20, 0.4 ml/100 ml of suspension, was added. The mixture was let stand overnight and then centrifuged at 3 x 10^4 x g. The supernate was used in the tests for heme synthesis. Test for synthesis was carried out as follows: In the cold sufficient solid potassium bicarbonate was added to the extract to make concentration 0.1 *M*. The mixture was gassed with 5% CO_2 in nitrogen for 5 minutes. In each of a number of 25 ml beakers was placed 28 micromoles of cysteine hydrochloride plus equivalent bicarbonate, 1 ml of solution containing about 250 millimicromoles of protoporphyrin(5), and 1 ml of solution containing a roughly equivalent amount of iron. Both solutions were prepared just before use. The iron, as ferrous ammonium sulfate alone or, when radioactive iron (Fe^{59}) was used, as a mixture of the sulfate and ferric chloride, was dissolved in 10^{-4} *M* hydrochloric acid plus 0.13 *M* potassium chloride. The porphyrin was dissolved in 0.015 *M* ammonium hydroxide plus 0.1 *M* bicarbonate. Shortly after addition of the other materials, a 12 ml portion of the gassed extract was added to each beaker. The test mixtures were then gassed with the carbon dioxide in a Dubnoff bath with shaking at 5° or less for 10 minutes. One half of each mixture was removed and treated for a given assay. The remainder under the gas was incubated at 37° for 30 minutes, then treated for the assay. The pH of the test mixture at room temperature initially and at the end of incubation was about 8.0, the optimum for heme formation(2). Inorganic iron in the samples of mixture was estimated by treating protein-free filtrates(6) with reductant and o-phenanthroline(7). Porphyrin and heme were extracted from other samples with acid ethyl acetate(8). The porphyrin was removed from the acetate with 3 *N* hydrochloric acid and assayed in an Evelyn colorimeter(9) standardized spectrophotometrically(10). After porphyrin was removed, the acetate, which contained the heme, was treated in one of the following ways: (a) It was extracted with 10 ml portions of 1 *N* ammonium hydroxide until heme appeared in the alkali. The portion of alkali containing heme and 2 further portions were combined. (b) It was mixed with a little water, heated to remove the ethyl acetate, and neutralized with alkali. The heme in the solutions obtained by (a) and (b) was converted to reduced cyanide hemochromogen and assayed in an Evelyn colorimeter standardized spectrophotometrically(11). (c) The solution of heme in the acetate was digested with sulfuric acid and peroxide and assayed for iron(7). (d) When radioactive iron was used, radioactivity of the ethyl acetate mixture or of the ammoniacal extract (*cf.* (a) above) was determined. Heme formation was estimated from this activity and initial levels of inorganic and active iron. Heme formation was also estimated from these levels and the activity recovered as hemin(2).

Representative *results* are given in Table I. As indicated by Exp. 1-9, the decrease in inorganic iron and in porphyrin during incubation of complete test mixtures were in fair agreement with each other and the increase in heme. In the absence of added porphyrin (*cf.* Experiment 7*b*), there was essentially no change in level of iron or heme during incubation or indication of porphyrin formation. Without added iron a little porphyrin disappeared (*cf.* Experiment 7*c*). This may represent heme formation since the test mixtures contained a little inorganic iron initially, some of which disappeared during incubation. As indicated earlier by use of labeled iron(1-4), no appreciable heme formation occurred in the cold, (*cf.* Experiments 4 and 5), with preparations heated to 100° (*cf.* Experiment 8), or in absence of the cell preparation (*cf.* Experiment 10).

The relatively direct estimates of heme

TABLE I. Synthesis of Heme from Protoporphyrin and Inorganic Iron in Presence of Extracts of Duck Erythrocytes. Experimental details given in text. In each experiment a different sample of blood was used for preparation of the extract. In Exp. 1, 5 and 8 the iron used contained Fe^{59} giving about 2.5×10^4 counts/min. An increase in amount of material designated +, a decrease −.

Exp.	Component of test mixture assayed	Amt of component present in 7 ml test mixture initially (mμmoles)	Change in amt of component/7 ml of test mixture during 30-min. incubation (mμmoles)	Estimates of heme used; exp. conditions other than indicated above
1	Porphyrin	235	−80	Heme estimated from Fe^{59} in ammoniacal extr. and in hemin.
	Iron	250	−64	
	Heme		+79	
	"		+78	
2	Porphyrin	248	−86	Heme from ethyl acetate estimated as hemochromogen.
	Iron	265	−88	
	Heme	1800	+120	
3	Porphyrin	214	−66	Heme from ammoniacal extr. estimated as hemochromogen.
	Iron	260	−71	
	Heme	1440	+40	
4	Porphyrin	240	−70 (+ 1)	Heme estimated as iron in ethyl acetate. Results in parentheses obtained with test mixtures kept at 2° for 30 min.
	Iron	262	−67 (+ 2)	
	Heme	1280	+80 (− 1)	
5	Porphyrin	241	−65 (− 2)	Heme estimated from Fe^{59} in ethyl acetate. Results in parentheses obtained with test mixtures kept at 2° for 30 min.
	Iron	259	−59 (+ 3)	
	Heme		+72 (+ 4)	
6	Porphyrin	242 (246)	−49 (−51)	Heme estimated from iron in ethyl acetate. Results in parentheses obtained with test mixtures that included preparation of globin(15).
	Iron	248 (252)	−45 (−46)	
	Heme	1350 (1370)	+50 (+50)	
7*a*	Porphyrin	221	−81	Heme estimated from iron in ethyl acetate. In *b* porphyrin omitted. In *c* iron omitted.
	Iron	245	−72	
	Heme	1770	+70	
b	Porphyrin	1	− 1	
	Iron	245	+ 3	
	Heme	1780	− 5	
c	Porphyrin	224	− 5	
	Iron	10	− 4	
	Heme	1770	0	
8	Porphyrin	242	−89 (− 2)	Heme estimated from Fe^{59} in hemin. Results in parentheses obtained with extr. heated to 100° for 7 min.
	Iron	258	−80 (+ 3)	
	Heme		+92 (+ 4)	
9	Porphyrin	239	−66	Heme estimated as iron in ethyl acetate. Cell preparation prepared by use of ammonium sulfate.
	Iron	250	−68	
	Heme	102	+71	
10	Porphyrin	235 (237)	− 3 (− 2)	Heme estimated as iron in ethyl acetate. Erythrocyte preparation omitted from test mixtures; comparable salt mixture + detergent was substituted. Results in parentheses obtained with mixtures containing globin.
	Iron	250 (255)	+ 2 (+ 4)	
	Heme	0	+ 1 (− 2)	

formation used in Experiments 2-4, 6, and 7 were of limited accuracy since the heme formed in these and similar tests did not exceed 8% of that present initially. Increase in the ratio of heme formed to initial heme was obtained with cellular material prepared as follows: In the cold, extract obtained as described above was mixed with 0.1 volume of 0.5 *M* potassium phosphate and 0.5 volume of saturated ammonium sulfate, each at pH 7.2. The resulting precipitate was collected by centrifugation, dissolved in a volume of .1 *M* potassium bicarbonate plus .156 *M* chloride equal to one-fourth that of the extract, and

tested as described above. The final solutions prepared by the sulfate treatment, were usually about as active as the extracts from which they were prepared; thus, about one-fourth of the activity was recovered. As indicated by Exp. 9, the ratio of heme formed to initial heme was increased by the sulfate treatment. Similar results were obtained using other estimates of heme. Although the concentration of hemoglobin, indicated by the heme content, in the solutions obtained by the sulfate treatment was less than in the extracts, the activity per unit of protein, as indicated earlier for the simpler cell preparation(2), was not sufficient to allow unequivocal assignment of the activity to a catalyst.

Discussion. The present results establish net synthesis of heme from protoporphyrin and ionic iron in the presence of material from duck erythrocytes and indicate, as reported earlier(1-4), that the material from the erythrocytes is necessary for the synthesis. However, it appears that under certain conditions(12-14), notably in the presence of a detergent and globin(14), heme is formed at neutral pH and moderate temperature without addition of cellular material. Under present experimental conditions globin had no effect on heme synthesis in the presence of red-cell extracts (*cf.* Experiment 6) and alone (*cf.* Experiment 10) did not promote synthesis. Nevertheless, it is possible that the material from the avian erythrocytes in some relatively non-specific way simply creates a suitable environment for heme synthesis.

Summary. Soluble preparations of duck erythrocytes were found to effect net synthesis of heme from protoporphyrin and iron.

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Repopulation of Hematopoietic Tissues of X-Irradiated Mice by Cells From Leukemoid Blood. (24815)

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Congdon *et al.*(1) reported that injection of blood showing extreme granulocytosis induced by a tumor prolonged survival of mice that had been given a lethal dose of total-body x-irradiation. Congdon *et al.* suggested that the leukemoid blood might protect against early death by some mechanism other than repopulation. The leukemoid blood might provide a humoral factor which would stimulate recovery of irradiated hematopoietic tissues or large number of leukocytes might help combat infection. Later Smith and Congdon(2) found evidence indicating that injected blood cells probably repopulated at least some hematopoietic tisues. More conclusive evidence that repopulation takes place is reported here.

The evidence was obtained by injecting leukemoid blood from mice of one genetic constitution into x-irradiated mice of another genetic constitution and later testing the irradiated, recipient mice by the Harderian-gland method(3) for presence of donor-type cells.

Materials and methods. The Harderian-gland test is based on the finding that although a nonvascularized graft survives in an homologous host because it does not initiate immunity, the graft is destroyed if host becomes immune(3). Injection of a suspension of homologous cells will immunize a mouse. In these experiments suspensions of cells from irradiated protected mice were injected into test mice of same genetic constitution as the irradiated mice. Each test mouse carried an Harderian-gland graft from a mouse of same genetic constitution as donors of the leukemoid blood. If tissues of irradiated mice contained cells that originated from the leukemoid blood, the grafts disintegrated. To provide a basis for estimating the proportion of cells originating from leukemoid blood, the immunizing capacity of a known number of cells of the same genetic constitution as leukemoid blood was compared with immunizing capacity of an equal number of cells from irradiated protected mice. Irradiated mice were (BALB/c X A) F_1 hybrids. The donors of the leukemoid blood were (BALB/c X C3H) F_1 hybrids. The following substrains were used in obtaining the hybrids: BALB/cAnN, A/LN and C3H/HeN. The tumor arose in a BALB/c mouse but was transferred for these experiments into (BALB/c X C3H)F_1 hybrids so that Harderian-gland grafts would come from a pigmented mouse. Disintegration of the pigment cells in the graft is the best end point for the Harderian-gland test. Squamous-cell carcinoma, A 280, used was kindly provided by Dr. Charles Congdon. He found that in mice supporting subcutaneous growth of this tumor, the leukocyte count was approximately 80 times normal in 4 to 5 months(1). In experiments described here blood was taken 6 months after mice were inoculated subcutaneously with the tumor. A half cc of blood was drawn from the heart of each mouse into a syringe moistened with saline containing heparin. The blood was immediately injected into the tail vein of a mouse that had been exposed, 1 to 6 hours previously, to 800 or 900 r total-body x-irradiation. Physical conditions for x-irradiation were: 200 KV, 15 ma, 126.5 r/minute at focus skin distance of 54 cm with .25 mm Cu and .55 mm Al filters. The irradiated mice were 3½ months of age and kept 3 or 4 to a cage. Three to 12 months after x-irradiation and injection of leukemoid blood, the mice were killed and certain tissues tested for presence of cells originating from leukemoid blood. Suspensions of marrow cells were obtained by pushing the marrow in Hanks' balanced saline through a 23 gauge needle several times. Other tissues were macerated in saline. The pH of saline was approximately 8.0 except in one experiment in which it was approximately 7.5. Dilutions were made so that about 100,000 cells could be injected in 0.5 ml volumes within approximately 30 minutes of time the suspensions were prepared. Further dilutions were made so that 25,000 cells could be injected in 0.5 ml approximately 30 minutes later. For comparative purposes similar preparations were made of cells from normal mice of same genotype as donors of leukemoid blood.

Results. Of 16 mice inoculated with tumor A 280, 2 showed no tumor growth at 6 months and leucocyte count was nearly normal. The 2 x-irradiated mice given blood from these animals died within 2 weeks, indicating no protection. Leukocyte counts for the 14 remaining animals ranged from 19,000 to 483,000/mm^3. Five irradiated mice received less than 50 million leukocytes, 4 received between 50 and 100 million and 5 received over 100 million. Six of these 14 mice were used from 3 to 12 months after treatment to determine origin of cells of certain of their tissues. Of the 8 other mice, 2 had died by 2 months after treatment, 5 more by 8 months and 1 more by 10 months.

A mouse tested at 88 days and one at 150 days gave positive tests for presence of homologous cells in marrow, lymph nodes, thymus, spleen and blood. A known number of marrow or lymph node cells from 4 other mice

TABLE I. Results of Harderian-Gland Test to Compare Antigenicity of Known Number of Cells from Irradiated Mice, (BALB/c × A)F_1, Protected by Leukemoid Blood from Mice of Another Genotype, (BALB/c × C3H)F_1, with Antigenicity of the Same Number of Cells from Mice with Same Genotype as Leukemoid Blood Donors.

Cells inj.		No. of test mice in which graft disintegrated/Total No. of test mice		
No. (×1000)	Tissue	Cells from irradiated mice protected with leukemoid blood	Cells from mice with same genotype as leukemoid blood donors	No. of days after irradiation
100	Lymph node	5/5		180
25		3/5		
100	*Idem*	3/6	3/6	218
25		2/6	2/5	
100	Bone marrow	2/3	3/3	271
100	Lymph node	4/4	4/4	370
25		2/4	2/4	

were tested at 180 to 370 days. Twenty-five thousand or 100,000 cells from the irradiated mice or from mice of the genotype of donors of leukemoid blood were injected into test mice. Preliminary tests with mice of genotypes used here as well as other experiments, some of which have been reported previously (4) with mice of other genotypes, showed that fewer than 25,000 homologous cells usually gave mostly negative tests. Further, with these numbers of cells a dilution of ¼ usually gave a detectable change in proportion of positive to negative tests. The results (Table I) indicate that at least ¼ and possibly almost all cells from marrow or lymph nodes of irradiated and protected mice were derived from the leukemoid blood.

Appearance of lymph nodes of the irradiated, protected mice at time the tests were made, varied greatly. The nodes were nearly normal in size and appearance in the 2 heaviest and most normal appearing mice, the mice killed at 150 and 218 days. The lymph nodes were smallest in the mouse that was least healthy, the mouse killed at 271 days. Almost no cells were freed when nodes from this mouse were macerated and virtually no lymphocytes were present in the node studied histologically. Tests had to be made on marrow cells from this mouse. In the rest of the mice, lymph nodes were smaller than normal and had yellowish opaque masses in them.

Discussion. Smith and Congdon(2) found that the general picture of recovery after irradiation and injection of leukemoid blood was the same as after injection of marrow. They believed that the cells of leukemoid blood repopulated at least the granulopoietic elements. The present results indicate that lymphocytes also are derived from leukemoid blood. Several studies have indicated that the lymphocytic cells in mice irradiated and given marrow are derived from the introduced marrow. One of these studies was made in this laboratory using the Harderian-gland test (4). Tests have been made on lymph node cells of 3 more mice at 358 to 450 days after irradiation and marrow injection. The antigenicity of 25,000 and 100,000 cells from protected mice was about the same as that for an equal number of cells from mice of the same genetic constitution as marrow donors. Thus the previously reported observations have been confirmed and extended.

Although in the present experiments, mice were protected from early deaths after irradiation by injection of homologous blood, Congdon *et al.*(1) and Smith and Congdon(2) were able to get prolonged survival with isologous blood only. The graft-against-host reaction, which seems to be important in length of survival of irradiated mice after injection with homologous hematopoietic tissues(5,6), may be less severe in the hybrid combination used here, because some of the components at the H2 locus are common to C3H and A strains (7). Further the genes that are different in the 2 hybrids are present in only ½ of the

dosage that would be found if 2 different strains were used. The possible role of dosage effect in increasing the protection afforded irradiated mice by injection of homologous cells is discussed by Uphoff(8).

Summary and conclusions. Survival of lethally x-irradiated (BALB/c X A)F_1 hybrid mice was greatly prolonged by injection of leukemoid blood from (BALB/c X C3H) F_1 hybrid mice. Quantitative estimates indicate that many, if not all cells in lymph nodes or marrow of irradiated protected mice were derived from leukemoid blood.

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Molecular Weight and Plasma Substituting Effectiveness of 3 Plasma Expanders. (24816)

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On the assumption that only compounds of high molecular weight were capable of substituting for blood plasma, dextrans of high molecular weight were initially employed in treatment of shock. These dextrans gave rise to a high incidence of side effects which were attributed to high molecular weight of the compound(1). Wilkinson and Storey(2) essayed a dextran considered to be "highly fractionated" (65% of molecules weighing between 130,000 and 250,000). They found in normal volunteers that it still produced a high incidence of urticaria and vasomotor instability. Since then the molecular weight of clinically used plasma expanders has been greatly diminished without significant change in their therapeutic effectiveness. Most of those now in use have an average molecular weight between 30,000 and 40,000(3). The study of a new compound of much lower molecular weight was considered interesting since it might produce fewer side effects than reported for the higher polymeric expanders. In the present investigation 3 plasma expanders were compared as to their capacity to restore normal blood pressure and plasma volume as well as to prevent death from an experimentally produced hemorrhage.

Materials and methods. The low polymeric plasma expander studied was a partially methoxylated polymer of galacturonic acid whose molecular structure resembles that of dextran (Fig. 1). Its average molecular weight was only 6,200. This substance was used in a stabilized and buffered 1% solution in saline (Graplasmoid, L. I. F. E.). The relatively higher polymers employed for comparison were: 6% dextran in saline (Intradex, Glaxo) with average molecular weight of 40,000, and

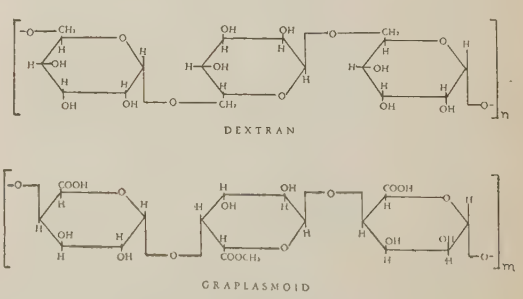

FIG. 1. Chemical structure of a moiety of dextran and Graplasmoid.

3.5% polyvinyl-pyrrolidone (PVP) in saline (Periston, Poulenc) with an average molecular weight of 28,000. As reference standard, heparinized whole blood obtained from test animal itself was used. For further comparison 2 crystalloid aqueous solutions, namely, 5% dextrose and 0.9% NaCl, were employed. All solutions were sterile and free of pyrogens. Since Rosenthal and Millican(4), in a review on the role of various fluids in traumatic shock and hemorrhage (287 references), conclude that there is no standard method for evaluating therapeutic value of plasma expanders and that tests in dogs are difficult to standardize because of lack of uniformity of race, weight, etc., in any adequately large population of animals, white male rabbits of a uniform strain weighing approximately 2 kg and guinea pigs weighing 0.5 kg were chosen for the present study. To determine the most appropriate amount of blood withdrawal ("hemorrhage"), preliminary experiments were conducted in which the animals were freely bled to death by opening one carotid artery and amount of blood shed was measured. Under these conditions average loss (± standard error), in 10 rabbits, was 30.6 ml/kg ± 0.98 ml/kg and 35.2 mg/kg ± 3.8 ml/kg, in 10 guinea pigs. In both species average bleeding time was 5 minutes. On the basis of these results 15 ml/kg (50% of the lethal blood loss in rabbits) was chosen as "standard hemorrhage." In series A, experiments in rabbits were performed after Hamilton *et al.*(5), by producing rapid standard hemorrhage (1 minute duration) from the carotid artery and replacing the blood lost by an equal amount of test fluid, injected immediately after hemorrhage (1 minute duration). The effectiveness of plasma expanders was evaluated by studying blood pressure over a carotid cannula for 2 hours, following experimental hemorrhage. All animals were heparinized, and anesthetized intraperitoneally with a combination of urethan (700 mg/kg) and pentobarbital (30 mg/kg). In series B, effectiveness of plasma expanders was evaluated by determining blood volume freely shed from opened carotid artery until death, 4 hours after standard hemorrhage and subsequent replacement of blood by the test fluid (*cf.* Lawson(6)). In additional groups, C and D, percent survival after partial replacement of blood by test fluid was studied. In series C, animals from series A were employed. After recording blood pressure for 2 hours, the carotid wound was sutured and the animal observed 24 hours. In series D, the standard hemorrhage and respective substitution of blood by test expander were made twice with an interval of 24 hours. Incidence of death was recorded for 24 hours after second hemorrhage. Studies on survival were also performed in guinea pigs (series E and F). After a severe hemorrhage of 30 ml/kg the volume of test expander injected was 10 ml/kg (⅓ of amount shed) in series E, while in series F it was equal to the amount shed (30 ml/kg). Survival was recorded for 24 hours after substitution of blood by the expander.

Results. (a) *Changes of blood pressure.* A rapid loss of 15 ml/kg of blood produced an abrupt fall of blood pressure 42 to 49% of normal. The immediate infusion of equal amount of any of the 6 test fluids rapidly restored blood pressure to near-normal levels (Fig. 2). Differences were not significant. This return, however, was followed during the next 10 minutes by drop in blood pressure, which varied greatly according to fluid used. When blood substitution consisted in autotransfusion, blood pressure, once restored after initial fall, did not essentially change. A

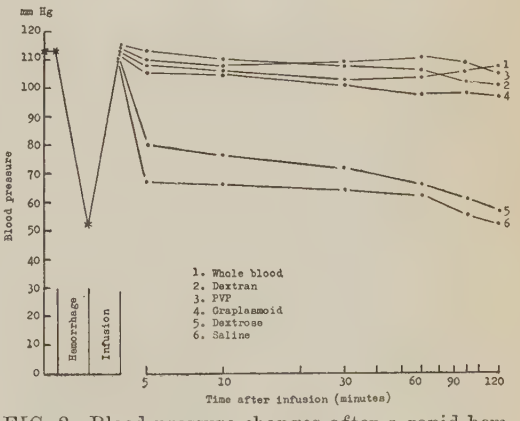

FIG. 2. Blood pressure changes after a rapid hemorrhage and an immediate infusion of a test fluid. Each point represents avg systolic pressure in groups of 8 to 15 rabbits.

slow decrease by about 10 mm Hg during the first hour was followed during second hour by complete return to original prehemorrhagic pressure. Behavior of blood pressure after dextran, PVP and Graplasmoid was not essentially different from that observed after a blood infusion. The final level after test period of 2 hours, was only slightly below that reached after blood. Even the largest difference, observed with Graplasmoid, the only one which was mathematically significant ($P = <0.05$) was not more than 15 mm Hg.

Quite different was the blood pressure curve after dextrose and saline. The rapid return to normal level after post-hemorrhagic drop was followed, within 5 minutes after infusion, by equally rapid drop by 35 and 45 mm, respectively. From this lower level the pressure continued to decline during 2-hour observation period, finally ending at the low post-hemorrhagic level. Differences from levels after blood infusion were significant ($P < 0.01$).

TABLE I. Blood Volume Obtained from Rabbits Freely Bled unto Death, 4 Hours after Replacement of 15 ml/kg of Blood by a Plasma Expander.

Plasma expander	No. of animals	Avg ± stand. error (ml/kg)	Relative vol
Blood	5	28.9 ± 2.2	100
Dextran	6	27.7 ± 2.0	96
PVP	5	27.2 ± 2.1	94
Graplasmoid	6	26.5 ± 2.3	92
Dextrose	5	17.9 ± 2.2	62
Saline	5	17.2 ± 2.3	60
Untreated controls	5	17.1 ± 2.1	59

b) *Blood volume 4 hours after infusion.* Blood volumes shed in a lethal hemorrhage 4 hours after partial substitution of blood by a test fluid are presented in Table I. Results obtained after infusion of plasma expanders were different from those after infusion of crystalloid solutions. While total volume shed after administration of test plasma expander was not essentially different from that after autotransfusion (only 8% lower in the case on Graplasmoid), it was greatly diminished after administration of dextrose or saline.

c) *Post-hemorrhagic survival.* In a group of non-treated rabbits 62.5% of animals survived 24 hours after a single standard hemorrhage. Survival diminished to 25% when blood withdrawal was repeated 24 hours later. When the blood shed was replaced by dextrose or saline, practically the same results as in non-treated group were obtained (Table II). However when blood was replaced by one of the 3 colloidal expanders, more than 90% of animals survived both single and double hemorrhage.

TABLE II. Survival of Rabbits 24 Hours after a Single (Group A) and a Double (Group B) Experimental Hemorrhage Treated with Various Fluids. Amount of blood replaced by an equal volume of plasma expander was 15 ml/kg.

Plasma expander	Group A		Group B	
	Death	% survival	Death	% survival
Blood	0/8	100	0/8	100
Dextran	"	"	1/10	90
PVP	"	"	0/10	100
Graplasmoid	1/15	93	"	"
Dextrose	3/10	70	5/7	29
Saline	2/8	75	6/8	25
Untreated controls	3/8	62	"	"

The results in guinea pigs, after severe hemorrhage (30 ml/kg) are presented in Table III. When 10 ml/kg of a colloidal fluid were injected immediately after the hemorrhage, 60 to 70% of animals survived, and when amount of expander infused was the same as blood withdrawn, survival increased to 100%. Substitution of blood by dextrose or saline was less effective in protecting animals against death.

Discussion. According to our experiments a polymer of low molecular weight (Graplasmoid) had a plasma substituting capacity qualitatively similar to that of polymers of

TABLE III. Survival of Guinea Pigs 24 Hours after a Hemorrhage of 30 ml/kg Treated with Different Doses of a Plasma Expander.

Plasma expander	Infusion = 10 ml/kg		Infusion = 30 ml/kg	
	Death	% survival	Death	% survival
Blood	1/5	80	0/5	100
Dextran	3/10	70	0/10	100
PVP	2/5	60	1/5	80
Graplasmoid	3/10	70	0/10	100
Dextrose	3/5	40	3/5	40
Saline	4/5	20	4/10	40
Untreated controls	5/5	0		

higher molecular weight, such as dextran and PVP. Quantitative differences were rather small and are possibly devoid of biological significance. It seems that if a plasma expander has characteristics of a colloidal solution, large weight of molecules might not be an indispensable factor in restoring hemodynamic properties of blood plasma. Nevertheless, molecular size and shape must be also studied before a definitive conclusion on the influence of molecular weight upon effectiveness of a plasma expander can be drawn.

It now seems(7,8) that high molecular weight of compounds is not the only factor involved in production of side effects. Wilkinson(7) found that, in spite of the fact that dextrans of 42,000 or 38,000 average molecular weight produced a lesser incidence of side effects than still higher polymeric compounds (2), the 42,000-type produced even a lesser incidence than the 38,000-type, a difference the author attributed to the fact that 2 different strains of *Leuconostoc mesenteroides* were used to synthesize the 2 dextrans and may have produced molecules of somewhat different structure. Moeller(9) in another kind of assay also found that dextran of lower molecular weight produced less cutaneous reactions.

Dextran is a mixture of polymeric hexoses in which 1:6-alpha-glucoside links prevail. Kabat and Berg(8) suggested that the differences between precipitin values produced by different dextrans could be related mainly to the number of 1:6 links, rather than to molecular weight. For another antigenic phenomenon, *i.e.*, complement fixation, however, Hehre and Neill(10) found it greater with heavier molecules. Dextran and PVP, besides their antigenic properties, induce liberation of histamine(11), to which some of the side effects are ascribed. It has been demonstrated(12) that histamine release also increases with higher molecular weight and concentration of dextran. Dextrans of molecular weight below 14,000 as well as dextran sulphate of molecular weight below 10,000 did not induce histamine release(12). Furthermore, Walton(13) showed that toxicity of dextran sulphate could be decreased by diminishing its molecular weight. The "reactions" to plasma expanders —allergic reactions, agglutination, complement fixation, precipitation, histamine release, etc.—seem to be rather complex; they appear to depend upon various factors such as weight, size, shape, and structure of molecule and concentration of drug. Like dextran and PVP, Graplasmoid is used therapeutically as a plasma expander and has not yet been reported to produce any side effects described for higher polymeric plasma expanders. Because of its low molecular weight a low concentration of Graplasmoid (1%) is required to obtain an isotonic solution with plasma, whereas higher concentrations of PVP (3.5%) and dextran (6%) are required. This low concentration could be a contributing factor in diminishing side effects.

Summary. Capacity of 3 plasma expanders of different molecular weight to substitute blood plasma was studied. These included dextran (40,000), PVP (28,000) and Graplasmoid (6,200); they were compared with whole blood and with 2 non-colloidal solutions dextrose and saline. Changes in blood pressure and blood volume observed after partial replacement of blood by any of the 3 colloidal solutions were not essentially different from those observed after autotransfusion. Replacement by dextrose or saline solutions, however, resulted in significant changes. The non-colloidal solutions only slightly delayed onset of hemorrhagic shock. The plasma expander with lowest molecular weight was as effective as the 2 high molecular weight compounds. A greater number of animals survived partial or "total" replacement of blood with plasma expanders than survived replacement with dextrose or saline. In percent of survival, no significant differences were observed between the 3 plasma expanders.

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Pituitary Prolactin Levels in Laying, Incubating and Brooding Pheasants (*Phasianus colchicus*).* (24817)

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The ring-necked pheasant (*Phasianus colchicus*) in Wisconsin can be classified as an "indeterminate layer"(1). This classification is used for birds which lay until accumulation of eggs releases the incubation response. Pheasants in Wisconsin drop a considerable number of eggs prior to laying in clutch and incubating(2). The physiological control of initiation of incubation and broodiness has been studied. Riddle and Lahr(3) and Lehrman(4) indicated that progesterone induces incubation in ring doves. Only recently has progesterone been identified in birds(5,6,7). Association of prolactin with broodiness is well known(8,9,10). Crispens(11) was able to induce broodiness in hen pheasants prior to and during reproductive season by injecting prolactin. His criterion for broodiness was acceptance of chicks by a treated hen. One can postulate that initiation of incubation is controlled by prolactin. Actual relationships between pituitary prolactin levels during incubation and brooding is not well established. Understanding of various physiological changes during the reproductive period is necessary before activities of bird can be properly interpreted. We conducted this study to determine pituitary prolactin levels during various stages of incubation and brooding.

Materials and methods. Hen pheasants were autopsied in various stages of reproduction in 1955 and 1956. Anterior pituitary glands of some of these birds were removed, weighed and immediately frozen for prolactin bioassay. In 1955, 3 to 5 glands were chosen from birds in the following stages: laying; 8th, 16th and 20th days of incubation; and 3rd, 7th and 11th days after hatching. The same procedure was followed in 1956 when 3 to 5 glands were chosen from birds in both laying and non-laying condition 4th, 8th, 12th, 16th and 20th days of incubation; and 3rd, 7th and 11th days after hatching. In late July, immediately following breeding season, glands from birds in each stage of reproduction were pooled and homogenized in a glass homogenizer. The homogenate was diluted with distilled water to concentration of 20 mg fresh weight tissue/cc. These aqueous homogenates were injected intracutaneously over crop glands of 8-week-old white king pigeons, a modification of technic of Lyons and Page(12). With this method it is possible to test 2 homogenates in each bird, 1 for each crop gland. Prior to assay homogenates were coded. Crop glands to test each homogenate, were selected at random. Each preparation provided test material for at least 2 assay glands. Water placebo was injected over 4 crop glands to provide additional control. The spot over each crop gland around

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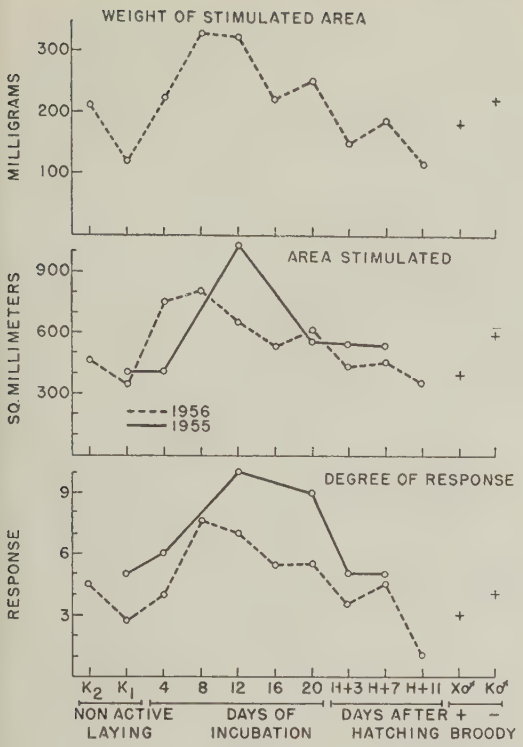

FIG. 1. Response of pigeon crop glands to pheasant pituitary homogenates from various stages of reproduction.

which injections were made was marked by injecting India ink. Injections of homogenates were made daily at noon for 4 days. Each crop gland received 0.125 cc/day and 4-day total of 10 mg of pituitary tissue. Each day's material was injected in a quartile clockwise around the marked site. Birds were autopsied on 5th day and gland response was determined in 3 different ways. First, degree of development was determined on 10 point basis. Absence of development was recorded as 0, initial development of rugae was rated 1 to 3. Additional thickening of stimulated area and production of pigeon milk were rated 4 to 10. Second, length and width of stimulated area of crop gland were measured by stretching the gland over base of small beaker and holding it up to the light. Sufficient tension was applied to keep tissue taut without tearing. Product of length and width of stimulated area is expressed as mm^2 of gland stimulated. Third, the stimulated area of crop gland was excised and weighed. Results were decoded following examination of crop glands at time of autopsy.

Results. Examination of Fig. 1 reveals that form of curves by all 3 criteria of crop response were essentially the same. Pituitary prolactin levels as measured by pigeon crop responses were lower for laying birds than for post reproductive non-laying controls. Prolactin content rises steadily during early incubation to a high level about 8th to 12th day. Responses from pituitaries of birds during late incubation and brooding, indicate a progressive decline from high mid-incubation level. The initial rise in pituitary prolactin during incubation can be interpreted as increased rate of formation by the pituitary. Broodiness in cock pheasants is a relatively rare occurrence. In 1956, however, we measured pituitary prolactin levels in 2 broody cocks as compared to 2 non-broody cocks. After 20 days incubation the pituitary prolactin levels in broody cocks were lower than those of non-broody cocks, as measured by our bioassay technic. These data suggest that pituitary prolactin levels do not continue to increase with advance of incubation, instead they tend to decline as incubation progresses.

Discussion. Godfrey and Jaap(13) demonstrated that broodiness in chickens can be terminated by estrogen. This could indicate that prolactin production or release is suppressed by high estrogen levels. The laying bird would normally be accepted as one in which estrogen titers were high. The commonly accepted mechanism in mammals, however, is one in which high estrogen levels stimulate production of prolactin. Our data indicate that a sudden acceleration in prolactin production occurs in response to internal or external stimuli. The stimulus necessary to elicit intense broodiness following this period of high prolactin production, may be considerably lower than in pre-incubation birds. The real expression of broodiness occurs in the latter half of incubation and after hatching, though individual differences exist between birds (Breitenbach, Meyer and Nagra, in manuscript). The action of prolactin on broodiness was first demonstrated by Riddle, Bates and Lahr(8). Many authors have since contributed information establishing correla-

tion between brooding behavior and prolactin stimulation. See Collias(14) for review and evaluation of the literature.

Relation of hormone production by bird pituitary to target organs and their secretions during reproduction is complex. Prolactin's inhibition of FSH production has been reported(15,16). The effects of progesterone in interrupting lay and initiating molt are counteracted by prolactin(17). Progesterone activity has been reported for extracts of avian blood(5,7) and in extracts of ovaries from laying fowl(6). Some workers reported effects of exogenous progesterone on bird physiology. Among them, Riddle and Lahr (3) showed that both progesterone and testosterone induce incubation and apparently stimulate release of prolactin by dove pituitary. Participation in incubation itself, stimulates prolactin secretion(18). Molt is characteristically inhibited during the brooding period (Breitenbach and Meyer, in manuscript). Inhibitory action of prolactin on molt process, reported by Juhn and Harris (17), is the probable explanation for delay in onset of molt. As brooding season progresses prolactin levels decline and the molt proceeds. Lehrman and Brody(19) demonstrated synergistic action of estrogen and progesterone on the dove oviduct and indicated that progesterone induces immediate incubation. Recently, however, in the turkey, it has been reported that progesterone seems to stimulate lay and interrupt broodiness(20). Obviously the relation of hormonal factors such as estrogen and progesterone to initiation of incubation is an area deserving more investigation.

Utilization of above information and our data tend to support the following hypothesis: Through accumulation of ovulated follicles in the ovary, increasing amounts of a substance (perhaps progesterone) are produced. This material causes production of prolactin by the pituitary. External stimulation is supplied by nest or accumulating eggs of clutch and is responsible for release and increased production of prolactin. The prolactin in turn inhibits FSH production and indirectly estrogen output. The influence of prolactin is expressed in broody behavior of the hen in relation to nest, and ultimately to the chicks.

Summary. Pituitary glands were removed from hen pheasants in various stages of reproduction—laying, non-laying, incubating and brooding and bioassayed for prolactin content. Prolactin levels rise rapidly during early incubation from levels observed in laying birds. Peak prolactin levels were noted from 8th to 12th days incubation. Prolactin content declined rapidly during latter portion of incubation and throughout the first 11 days following hatching. This occurred despite the fact that late incubation and post-hatching are periods of most intense brooding activity. Non-laying birds showed slightly higher pituitary prolactin levels than did laying birds. Pituitaries of 2 broody cocks were examined after 20 days incubation and showed less prolactin than pituitaries of 2 non-broody control cocks. It is suggested that early high prolactin levels condition the bird for subsequent brooding stimuli. The relationship to control mechanisms is discussed.

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Mammalian Cell Cultures for Study of Influenza Virus. I. Preparation of Monolayer Cultures with Collagenase.* (24818)

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Enzymatic treatment of cells, developed(1) for routine passage of cells in continuous culture, has been employed widely for primary dispersion of cells from tissue. While parts of some tissues, notably renal cortex, are dispersed readily by application of trypsin, other tissues are more or less refractory. Consequent restriction of cell types that can be dispersed efficiently by trypsin from tissue hampers *in vitro* study of some viruses, comparison of virus susceptibility of cells *in vivo* and in primary culture, and direct clonal derivation of cell lines from tissue. This paper describes the use of collagenase for preparation of primary monolayer cultures of lung, a tissue usually poorly dispersed by trypsin, for study of influenza viruses.

Materials and methods. Solutions included a) GKN (solution of glucose, potassium chloride and sodium chloride in concentrations appropriate to Hanks' solution)(2); b) Hanks' balanced salt solution brought to pH 7.4-7.6 with sodium bicarbonate; and c) nutrient medium containing 20% serum of the same species as the tissue cultivated in YEM (3) with penicillin and streptomycin added to final concentrations of 50 units and 100 μg per ml. *Tissues* were obtained as a) human and swine lungs from fetuses aged approximately 80 (14 cm) and 70 days respectively, and b) lung from an adult American Dutch rabbit about 250 days of age. Fetal swine lungs were obtained from miniature swine developed for research(4), and from sows randomly selected at an abattoir (by kind cooperation of Dr. Eldon G. Hill of the Hormel Institute, Univ. of Minnesota). Animals were opened aseptically and lung or other tissue removed to sterile Petri dishes containing about 2 ml of Hanks' solution. *Enzymes* in GKN were sterilized by passage through Selas #02 filters. Collagenase (Worthington Biochemical Corp., Freehold, N. J.) was prepared in stock concentration of 0.1% for use as 100 μg per ml (0.01%); trypsin as Difco Yeastolate was prepared in stock concentration of 20 mg per ml (2.0%) for use as 2 mg per ml (0.2%). *Preparation of dispersed cell suspensions. Lung* tissue was minced into 1-2 mm fragments cut by crossed scalpel blades handled like scissors; portions of bronchial tree separable from parenchyma were discarded. Fragments were rinsed repeatedly with Hanks' solution to remove erythrocytes, transferred to a screw-capped flask containing a Teflon-covered magnetic stirring bar, covered to an approximate depth of 30 mm with collagenase solution, and incubated for 10 min in a water bath at 37°C. Flask contents then were agitated by magnetic stirring for 2 hours in a 37°C incubator. An essential precautionary measure to prevent cell damage from heat was the insertion of an inverted Petri

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‡ This work was done in partial fulfillment of requirements for Ph.D. degree in Bacteriology and Immunology, Univ. of Minnesota.

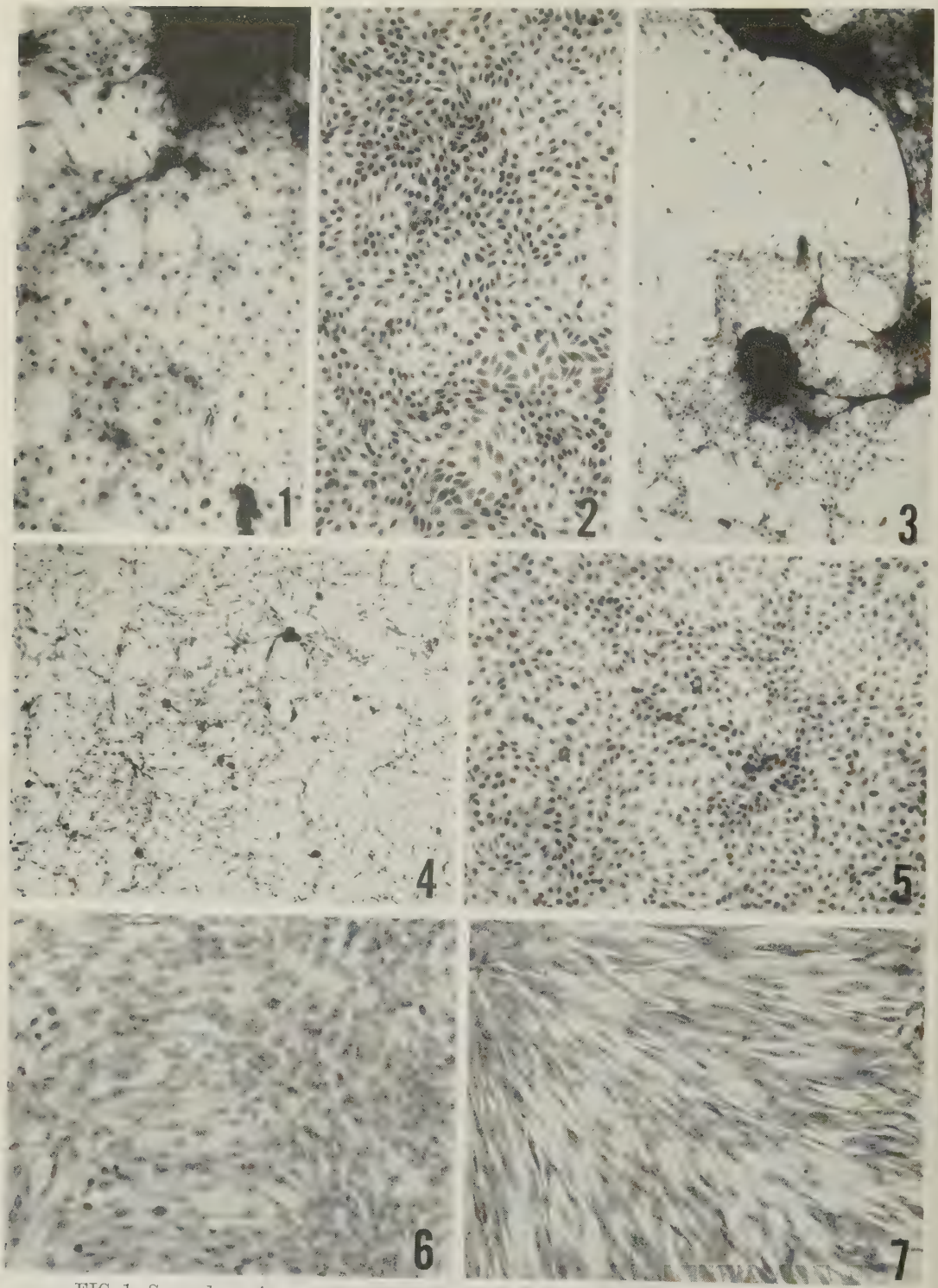

FIG. 1. Seven-day primary culture of porcine lung digested with trypsin; outgrowth extending from tissue fragment on right. ×120.

FIG. 2. Four-day primary culture of porcine lung dispersed with collagenase. ×120.

plate between flask and the Magnestir plate (Labline Inc., Chicago). After this treatment, usually sufficient to disperse the fragments, the resulting cell suspension was filtered through 4 layers of sterile gauze, sedimented at 20 x g for 6 minutes, separated from supernatant fluid and resuspended directly in nutrient medium. *Fetal kidney* cell suspensions were prepared similarly; collagenase dispersed both cortical and medullary tissue. With blood-engorged tissue, collagenase treatment was preceded by agitation of tissue fragments with trypsin for 2-10 hours at 5°C. After trypsin treatment, supernatant fluid was discarded from settled fragments and collagenase treatment carried out as previously described. Initial trypsinization reduced concentration of erythrocytes in the cell suspension, and reduced fragment size to facilitate more efficient collagenase action. *Monolayer primary cell cultures* were prepared from cell suspension in nutrient medium diluted to contain 10^6 cells per ml according to hemocytometer count(5) or diluted 1:150 according to packed cell volume(6). Screw-capped 16 x 125 mm culture tubes or 200 ml rectangular bottles were seeded with 1.0 ml or 10 ml amounts of suspension, respectively. After preliminary incubation at 37°C for 24-48 hrs, cultures were rinsed and the nutrient medium completely renewed. After 1-4 days of additional incubation, cell monolayers were ready for use. Subsequent dispersal of cells for transfer was accomplished by trypsin treatment.

Results. Adult or fetal lung tissue was found refractory to treatment with 0.2% trypsin in GKN or Hanks' solution. Agitation with trypsin at 37°C or overnight at 5°C, commonly sufficient to disperse monkey kidney tissue efficiently, produced fragments of lung about 0.2-2.0 mm in size. These course dispersions yielded short-lived outgrowth (Figs. 1, 3). In contrast, collagenase digested lung completely to liberate cell suspensions generating healthy monolayers in primary culture. After 3-4 days of incubation, monolayers were confluent and ready for use in virus studies (Figs. 2, 4). Unlike primary cultures prepared from trypsinized tissue, cells from collagenase-dispersed suspensions were readily passed by conventional trypsinization. Continuous cultures are shown in the 5th to 9th passages (Figs. 5, 6, 7). Cells in continuous swine lung cultures have the morphological appearance of mesothelial cells, while those of rabbit lung cultures appear more fibroblastic. Human lung in primary culture appeared to contain a mixture of fibroblastic and epithelial cell types; after 9 serial passages, cells appear uniformly epithelial. Effectiveness of trypsin and collagenase for dispersion was compared also with adult and fetal kidney tissue. Medullary tissue proved refractory to trypsin digestion but not to collagenase treatment.

Discussion. Commercially prepared collagenase was found an effective dispersing agent for preparation of monolayer primary cultures from lung and renal medulla, as examples of tissue difficult to disperse efficiently with trypsin. Cultures of lung tissue were of particular interest for study of influenza viruses.

Methods for dispersal of cells from tissue or culture monolayers have been instrumental in advancement of cell culture technics for study of viruses. Trypsin and versene have been widely and successfully employed for dispersion of embryonic or adult soft tissue, or cultures(1,7). Tissues relatively rich in connective tissue, however, have been difficult to disperse with trypsin(8). Since collagen fibers are basic elements of most types of connective tissue, collagenase was attractive as a dispersing agent. Lasfargues(9,10) obtained

FIG. 3. Two-day culture of trypsin-digested porcine lung. ×40.

FIG. 4. One-day culture of collagenase-dispersed porcine lung. ×40.

FIG. 5. Five-day culture of porcine lung cells in 5th serial passage by trypsin dispersal, from primary culture prepared from collagenase-treated lung. ×120.

FIG. 6. Six-day culture of human lung cells in 9th serial passage by trypsin dispersal, from primary culture of collagenase-treated lung. ×120.

FIG. 7. Six-day culture of rabbit lung cells in 6th serial passage by trypsin dispersal, from primary culture of collagenase-treated lung. ×120.

short-lived cultures from parenchymal fragments of mouse mammary gland released by exposure to collagenase. The present studies employed glucose-potassium chloride-sodium chloride solution as diluent for collagenase rather than Hanks' or other such phosphate-buffered solutions, because the enzyme has been reported unstable in phosphate buffer (11). Present experience indicates that the commercial enzyme in stock solution is stored better at 5°C than frozen, and that best results are obtained when enzyme solutions in GKN are prepared shortly before use. Although prior digestion of lung tissue with trypsin often was advantageous to reduce erythrocyte contamination, well dispersed cell suspensions were prepared with collagenase alone. The purified bacterial enzyme as commercially prepared may include sufficient proteolytic enzyme, in addition to collagenase, to separate cells released from collagen-containing stroma. Use of collagenase(12) to release cells in quantity from tissue parenchyma extensively interlaced with connective tissue can be expected to make available new cell types for diagnosis of viral infection, for wider study of virus-host cell relationships, and for new sources of virus vaccine. Use of the enzyme makes available a convenient system for study of swine influenza virus, in particular, since porcine fetal lung together with a supply of serum can be obtained from local abattoirs.

Summary. Primary monolayer cultures of human, rabbit and porcine fetal and adult lung were prepared conveniently by dispersion of tissue with commercially available collagenase in phosphate-free salt solution.

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Mammalian Cell Cultures for Study of Influenza Virus. II. Virus Propagation.* (24819)

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Cells easily dispersed from continuous cultures or from particular tissue sources such as kidney and amnion are the mainstay of cell culture procedures for detection and assay of viruses and antibodies. For specific study of host-cell relationships, however, it is desirable to work with primary and/or continuous cultures of cell types known to be affected *in vivo*. This paper describes susceptibility to human and swine influenza viruses of primary cultures of cells released from lung parenchyma by treatment with collagenase.

Materials and methods. Cultures of human, rabbit and porcine lung, and whole por-

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cine kidney for comparison, were prepared as previously described (paper 1). *Virus sources* included 14th egg-passage Asian A influenza virus obtained from Dr. Marvin Field, Pfizer Laboratories, Terre Haute, Ind.; the PR-8 strain of influenza A (T. Francis, Jr., passaged in ferrets 34x, mice 593x, eggs 165x), and Lee strain of influenza B, (both by purchase from the American Type Culture Collection, Washington, D.C.) passed 6 times in eggs before use; and swine influenza virus (#1976) kindly supplied by Dr. Morris Schaeffer, Communicable Disease Center, Montgomery, Ala. Virus stocks represented 1:10 dilutions of lyophilized material (stored at −70°C) in Hanks' solution supplemented with 5% horse serum. *Virus assays* with cell cultures were performed by inoculation of 5 culture tubes per 10-fold serial dilution and calculation of cytopathogenic 50% endpoints by the method of Reed and Muench(1). For similar calculations of egg infectivity titers (EID_{50}), allantoic sacs of 10-day embryonated eggs were inoculated with 0.1 ml doses of virus dilutions; eggs were incubated at 35°C until infectivity was apparent or for a period not exceeding 96 hours. Hemagglutinating potency of virus material was determined by standard procedure(2). Propagated influenza viruses were identified by use of hyperimmune ferret serum (kindly supplied by Dr. Morris Schaeffer) in the Salk(3) hemagglutination-inhibition pattern test.

Results. To test susceptibility of primary human, rabbit and porcine lung cultures, confluent monolayers were washed free of nutrient medium with Hanks' solution, and re-covered with 0.9 ml of maintenance solution(4,5) containing 5% inactivated serum (homologous for culture species and free of influenza virus antibody) and penicillin and streptomycin to final concentrations of 200 μg per ml. Prepared tube cultures were inoculated with 0.1 ml of virus of known titer and incubated slanted and stationary at 37°C. For serial virus passage, inoculated culture tubes were frozen and thawed consecutively 3 times by immersion in alcohol-dry ice mixture, and inoculated into new cultures. Inoculated cultures were passed when cytopathogenic damage affected 70-80% of each monolayer, or after 6-7 days of incubation if cell destruction was minor. Human lung cultures unequivocally propagated A and B human influenza viruses and virus hemagglutinins despite cumulative dilution of original inoculum to insignificance (Table I). All 3 human influenza viruses retained egg infectivity after 15 passages in cell cultures. Contrariwise, infectious and hemagglutinating activity of swine influenza virus was lost rapidly with control serial passage. Cytopathogenic effect of the human influenza viruses on the human lung cells was minimal: peripheral rounding of monolayer cells, usually first observed 48-72 hours after virus inoculation, progressed for an additional 48-72 hours, but no further (Fig. 1). Identity of propagated human viruses was confirmed by hemagglutination-inhibition titers with specific antiserum (Table I).

Porcine adult and fetal lung cultures propagated swine influenza virus and hemagglutinin to high titer through 20 serial passages (Table I) despite theoretical extinction of original inoculum. Although only low cytopathogenic titers and no hemagglutinins were recorded for A strains of human influenza virus serially passed in porcine lung cultures, evidence of propagation was substantiated by final harvest of low-titer egg infectious virus. Results with influenza B virus were equivocal: minimal cytopathogenic effect persisted despite high cumulative dilution of original inoculum, but hemagglutinating activity was not found after 15 passages and egg infectious virus was not detectable. In contrast to behavior of viruses in human lung cultures, cytopathogenic effect of swine virus in swine lung cultures was marked after 48 hours incubation at 37°C (Fig. 2), and almost complete destruction was seen after 72-96 hours.

Rabbit lung and porcine kidney cultures were uniformly unresponsive to blind passage of human influenza viruses. Porcine kidney cultures, prepared from mixed cortical and medullary tissue, propagated swine virus hemagglutinin and yielded egg infectious virus after 15 passages, although cytopathogenic effects were not seen.

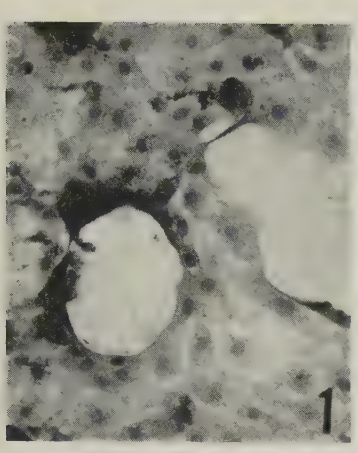
Discussion. For both epidemiologic and

TABLE I. Propagation of Influenza Viruses in Monolayer Cultures of Mammalian Lung Dispersed with Collagenase.

Cell culture source	Influenza virus	Passage No.	Total days in culture	Cumulative dilution of original inoculum (log)	Cytopathogenic titer (log $TCID_{50}$)	Supernate hemagglutination titer, 1:	Hemagglutination inhibition titer as reciprocal of initial serum dil.	Egg infect. titer (–log EID_{50})
Human lung	A (Asian)	0					NT	>5
		1	4	2	1.0	40	"	
		5	24	7	3.2	80	"	
		10	54	12	2.8	80	"	
		15	79	17	2.9	80	1280	5.2
	A (PR-8)	0					NT	>6
		1	4	2	3.3	20	"	
		5	24	7	1.4	30	"	
		10	54	12	2.0	40	"	
		15	79	17	2.4	80	512	3.8
	B (Lee)	0					NT	>4
		1	5	2	2.4	80	"	
		5	35	7	1.9	20	"	
		10	65	12	3.0	80	"	
		15	95	17	2.8	160	256	5.6
			Swine virus results were negative.					
Rabbit lung			A (Asian), A (PR-8), B (Lee) and swine virus results were negative.					
Porcine lung	A (Asian)	0					NT	>5
		1	4	2	neg.	10	"	
		5	24	7	1.2	neg.	"	
		10	54	12	2.0	"	"	
		15	79	17	1.8	"	"	1.0
	A (PR-8)	0					NT	>6
		1	4	2	neg.	neg.	"	
		5	24	7	"	"	"	
		10	54	12	1.3	"	"	
		15	79	17	1.0	"	"	1.6
	B (Lee)	0					NT	>4
		1	4	2	2.3	20	"	
		5	24	7	1.5	10	"	
		10	54	12	1.3	10	512	
		15	79	17	1.0	neg.	NT	neg.
	Swine	0					NT	>5
		1	4	4	6.5	160	"	
		5	19	12		640	"	
		10	34	22	7.9	640	"	
		15	49	32		320	"	
		20	65	42	8.0	640	512	8.4
Porcine kidney (cortex & medulla)			A (Asian), A (PR-8) and B (Lee) virus results were negative.					
	Swine	0					NT	>5
		1	4	2	neg.	40	"	
		5	27	9	"	20	"	
		10	51	14	"	20	"	
		15	80	20	"	20	"	2.0

NT signifies not tested.

fundamental studies of host-cell virus relationships, cultures of cells normally the target of *in vivo* infection are desirable. Although poliovirus notably has been studied successfully with cells not susceptible *in vivo*(6), other viruses including influenza viruses have not been so easily propagable in cultures of heterologous relationship(7,8,9). In these studies, human and swine influenza viruses clearly multiplied in and were usefully cytopathogenic for primary cultures of human and porcine lung, respectively. These findings emphasized the value of collagenase treatment for dispersal of tissues held together by



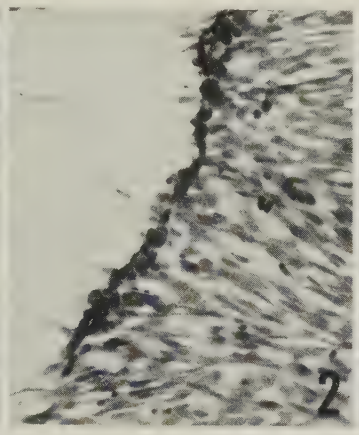


FIG. 1. Porcine lung cells of primary culture of collagenase-dispersed tissue, 48 hr after inoculation of swine influenza virus. ×200.

FIG. 2. Human lung cells of primary culture of collagenase-dispersed tissue, 96 hr after inoculation of human influenza virus. Peripheral cells are typically rounded. ×120.

FIG. 3. Rabbit lung cells of primary culture of collagenase-dispersed tissue, 6 days after inoculation of swine influenza virus. ×120.

fibrous stroma. Interestingly, specific host-cell virus relationships were disclosed. Swine influenza virus, for example, was propagated readily in swine lung cells but not swine kidney or human lung cells. The human Asian, PR-8 and Lee viruses multiplied in human lung cultures, and appeared to multiply also in porcine lung cultures, although cytopathogenic titers did not increase with serial passage and hemagglutinins were lost finally. Although propagation of human viruses in porcine lung cultures was of lesser magnitude (or equivocal in the case of Lee virus), it was sufficient to merit further investigation. If human strains could be adapted to better growth in swine cells without loss of antigenic character, a non-primate cell culture source of virus for human vaccine might be made available. Initial results with swine virus and porcine lung cultures are adequate to indicate that adult or fetal collagenase-dispersed lung should be useful for detection and propagation of this virus. The system appeared susceptible to quantitation because plaque formation was seen in infected monolayers, even without agar overlay. Although cytopathogenic effect of human influenza viruses on human lung cells was much less severe than that of swine virus on swine lung cells, the former as well as the later was neutralized specifically by diagnostic hemagglutination-inhibiting antiserum. The relative susceptibility to influenza viruses of human and porcine lung cultures, and presence of morphologically recognizable epithelial elements, suggest that collagenase-dispersal released parenchymal cells in large numbers. Although the same result might be expected for rabbit lung, it is not apparent whether the complete insusceptibility of collagenase-dispersed rabbit lung cultures to influenza viruses (Fig. 3) should be attributed to species or cell type.

Summary. Collagenase-dispersed human and porcine lung cells in primary culture unequivocally propagated human strains A and B, and swine influenza viruses, respectively. Swine lung cells also propagated human influenza A viruses to some extent. Swine virus infection destroyed, and human virus infection less severely damaged porcine and human lung cells respectively. Rabbit lung cells were insusceptible to human and swine influenza viruses. Swine kidney cells propagated egg-infectious swine influenza virus and hemagglutinin without cytopathogenic effect.

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Metabolism of Acid Mucopolysaccharides of Rabbits with Serum Sickness.* (24820)

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The clinical description of serum sickness by Von Pirquet and Schick(1), aroused considerable interest. More recently, Gregory and Rich(2) demonstrated that serum sickness can be produced readily in rabbits and can serve as a useful experimental model to study phenomena of hypersensitivity. In studies with rabbits, lesions of connective tissue structures are observed, similar to those associated with the group of diffuse diseases of human connective tissue, as lesions associated with polyarteritis nodosa. One factor that seems common to the human diseases, experimental serum sickness, and related hypersensitivity states is the similar pathologic involvement of matrix or ground substance of connective tissue. This similarity is the basis for considering an etiologic relation between hypersensitivity and diseases of connective tissue. Although such relation remains to be established, the inflammatory response of components of connective tissue to disease due to hypersensitivity is provocative. For purposes of studying this inflammatory response, biochemical technics were used to investigate components of the matrix of connective tissue. The effect of serum sickness on acid mucopolysaccharides (MPS) of skin and cartilage of rabbits was observed.

Methods and materials. Six groups of 4 to 6 rabbits, each of approximately 2.6 kg weight, were studied. The rabbits were offered water and laboratory chow freely. Serum sickness was induced in one-half or more of each group by 2 intravenous injections of horse serum (10 ml/kg) given 2 weeks apart (3). From each group 2 rabbits were selected: one demonstrating most clinical evidence of serum sickness, judged by sedimentation rates and changes in weight, was matched approximately by weight with a normal rabbit. Rabbits were sacrificed 6 days after second injection of horse serum, when maximal lesions from serum sickness are expected(3). Examination of gross and histologic sections† confirmed the presence of pathologic lesions of serum sickness. During period of observation after second injection of horse serum to sick animals, the pair selected from each group (one animal with serum sickness and its control) received subcutaneous injections (50 μc) of C^{14} carboxyl-labeled sodium acetate‡ in 0.15 *M* solution 3 times a day. One series of 3 pairs received the isotope for 3 days (450 μc) and another 3 pairs for 5 days (750 μc) before sacrifice. Timing of administration of labeled acetate was chosen for comparison with other observations in normal animals(4). MPS, hyaluronic acid and chondroitin sulfate, were isolated from skins of rabbits by methods previously described(5), and chondroitin sulfate was isolated from cartilage(6) obtained from various sites of body, such as ears, trachea and sternal cartilages. The amount of MPS isolated was determined by weighing or estimated from solutions by uronic acid deter-

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minations(7). In addition, glycogen was isolated from skeletal muscle and liver of individual rabbits and fractionated with alcohol (8) to yield preparations with optical rotation $[\alpha]_D^{23}$ of $+200°$. Radioactivity of C^{14} in MPS and glycogen was determined by microcombustion of samples, collected as $BaCO_3$ and counted in open window, gas-flow counter. Radioactive counts were corrected to "infinite thickness" and to equivalent weights of rabbits. Analyses of data from normal animals in these and other experiments (4) yielded average variation of approximately $\pm$ 18% (range, 1 to 47%) for a single animal. These experiments include triplicate determinations for each category studied.

Results. Total amount of MPS that could be isolated from skin or cartilage was essentially the same for each group of animals. Radioactivity of hyaluronic acid from skin was slightly higher for rabbits with serum sickness [avg. 3800 (3050-4180)] than for controls [avg. 3005 (2900-3165)] in the series that received isotope for 5-day period. Similarly, radioactivity of chondroitin sulfate from skin of rabbits with serum sickness [avg. 1603 (1330-1785)] was slightly greater than observed for controls [avg. 1324 (902-1600)] after receiving C^{14} acetate for 5 days. No apparent effect was observed for the series with 3 days of isotope administration and no differences of isotope incorporation were observed for chondroitin sulfate of cartilage in either series. In glycogen samples, a considerably greater incorporation of isotope in diseased animals was demonstrated. Increase of radioactivity in glycogen was noted particularly in samples from liver and in animals that received isotope for 5 days. The average of the group with serum sickness was 2080 (1440-2720) and 192 (171-211) for the control group. A 2-fold increase of comparative radioactivity for sick animals was observed for muscle. Smaller but definite differences in the same direction were found in the series receiving isotope for 3 days. In addition, total amount of glycogen that could be extracted from livers of rabbits with serum sickness was approximately one-half that for control animals.

Discussion. The magnitude of differences observed, is only slightly greater than the limit of error for such experiments. Since isolation and study of radioactivity of these compounds require a relatively large quantity of tissue, skin and cartilage were considered most suitable for study; however, selection of tissue may have limited the detection of changes of MPS. Serum disease produces diffuse pathologic changes, with small lesions throughout various connective tissue structures and tissues studies probably represent an admixture of tissue with and without lesions. These studies primarily measure incorporation of an isotope into MPS, without a quantitative estimate of the MPS pool. Although it would be desirable to know effect of serum sickness on rate of synthesis of MPS of the body or in a uniform lesion, incorporation alone is not an absolute measure of synthesis. The isolation under comparable conditions of equal amounts of MPS from tissues of normal and diseased animals and the slight variance of isotope labeling, suggest that serum sickness does not produce significant changes in the over-all metabolism of MPS.

Contrary to studies of MPS, a rather remarkable effect was observed on liver glycogen and, to a lesser extent, on muscle glycogen. That changes occurred in glycogen is a further manifestation of the generalized illness. Such changes in glycogen could be interpreted as nonspecific, being similar to those that accompany starvation(9,10).

Summary. Studies of incorporation of C^{14}-labeled acetate into MPS, hyaluronic acid and chondroitin sulfate from skin and chondroitin sulfate from cartilage of rabbits with and without serum sickness, indicated little difference effected by serum sickness. Specific activities of skin MPS were possibly increased. Greater incorporation of the isotope was observed in liver and muscle glycogen from rabbits with serum sickness. Further study of other hypersensitivity reactions and of tissues more specifically involved by such reactions need investigation. Such experiments, currently in progress utilizing the local Shwartzman lesion, do indicate a considerable increase of metabolic activity of the MPS.

The author is very grateful for cooperation of Dr. Albert Dorfman, in whose laboratory these experiments were begun.

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Serum Globulins in Experimental Nephritis. Effect of Conditioning Factors.* (24821)

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It has been shown that Masugi's experimental nephritis can be enhanced and evolution towards specific clinical forms determined with the aid of conditioning factors such as saline-drinking water and/or unilateral nephrectomy(1,2). The present paper is concerned with the effect of these conditioning factors upon various electrophoretic fractions of serum globulins in normal and nephritic rabbits.

Materials and methods. A. *Control*: Thirty-four albino rabbits of either sex weighing 2800-3200 g, were separated into 4 groups as follows: 16 were controls; 7 were submitted to unilateral nephrectomy; 7 were maintained on saline-drinking water; 4 were unilaterally nephrectomized and one month subsequently were given saline-drinking water. All animals were bled at various intervals during following 6 months. Total serum proteins were determined with biuret method of Reinhold(3). Tiselius moving boundary electrophoresis determined various serum protein fractions. The results were averaged for each group and mean values and standard deviation determined $\left(\text{S.D.} = \sqrt{\frac{\Sigma(X^2)}{N-1}} \right)$. B. *Glomerulonephritis* was induced with duck antirabbit-kidney serum prepared according to method previously described(2). Pools and amount of sera provoked clinical and pathological symptoms of glomerulonephritis in all experimental animals. However, in contrast with the kaleidoscopic but relatively mild symptoms of controls, the unilaterally nephrectomized group uniformly showed acute glomerulonephritis with early death accompanied by non-edematous (dry) uremia, while saline treated animals developed either subacute edematous or chronic polyuric glomerulonephritis. Clinical findings were substantiated by histologic studies(2). All animals were bled at varying intervals before and after nephrotoxic injection. The results of electrophoretic determinations, obtained between 7th and 21st day following nephrotoxic injection, were averaged and differences from control baseline expressed in standard deviation units.

Results. Table I shows results of 44 electrophoretic determinations averaged for each group of normal and preconditioned rabbits. Conditioning procedures do not alter the electrophoretic pattern of serum as compared to controls. Total mean values shown in extreme right column were used as control baselines for each electrophoretic protein fraction.

* This investigation was supported in part by Research Grant from U.S.P.H.S.

TABLE I. Electrophoretic Fractionation of Globulins in the Serum of Normal and Preconditioned Rabbits (mg/100 ml Serum).

Globulin fraction	Normal control	Unilaterally nephrectomized	Saline drinking	Unilateral nephrectomy and saline	Total mean ± S.D.
Total alpha globulins	1.01	1.09	1.04	1.00	1.03 ± .19
Alpha-1	.21	.28	.22	.21	.23 ± .09
" -2	.45	.44	.45	.44	.45 ± .09
" -3	.35	.36	.37	.35	.35 ± .09
Total beta globulins	.86	.90	.88	.84	.87 ± .22
Beta-1	.56	.62	.63	.62	.59 ± .16
" -2	.29	.28	.25	.23	.28 ± .15
Gamma globulins	.64	.70	.66	.60	.65 ± .24

Fig. 1 summarizes results of 25 electrophoretic determinations performed between 7th and 21st day following injection of nephrotoxic serum. The columns show average deviations from control values as expressed in standard deviation units (S.D.). Shaded area covers 2.6 S.D. (*c.g.*, P = .01).

The dose of nephrotoxic serum did not induce significant alteration in serum globulin values of control rabbits. However, unilaterally nephrectomized rabbits increased alpha-3 globulin fraction, while saline-drinking animals increased alpha-2 serum globulin component following nephrotoxic injection.

A typical normal electrophoretic pattern of rabbit serum is shown in Fig. 2. The additional intermediate fraction situated between alpha-2 and beta-1, has been conventionally called alpha-3. It was present in all 69 electrophoretic determinations.

Fig. 3 shows electrophoretic patterns of a control and of 3 preconditioned rabbits 3 weeks after intravenous injection of similar doses of nephrotoxic serum. Alterations of

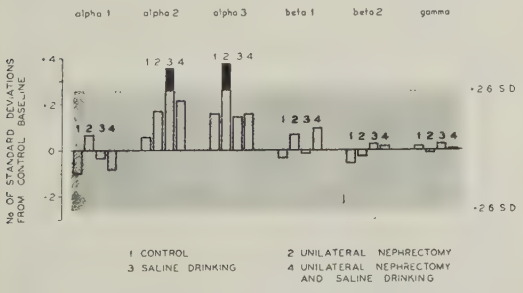

FIG. 1. Deviation from control baseline of various serum globulins during experimental nephritis induced with antirabbit kidney duck serum in rabbits: 1. normal; 2. uninephrectomized; 3. saline-drinking; 4. uninephrectomized and saline-drinking.

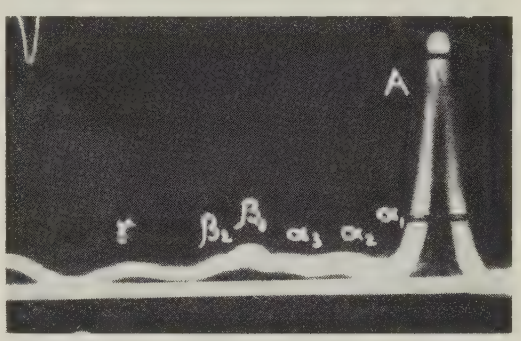

FIG. 2. Moving boundary electrophoretic pattern of normal rabbit serum.

serum protein patterns are in agreement with average deviation shown in Fig. 1.

Discussion. From above results it may be concluded that nephrotoxic sera, in doses that do not influence the electrophoretic serum globulin pattern of control rabbits, may induce significant alterations when given to animals preconditioned with saline and/or uninephrectomy. When nephrotoxic serum was given to saline preconditioned animals, a significant increase of alpha-2 serum globulin was noticed. Considering that these animals developed chronic glomerulonephritis with polyuria and hyposthenuria(1,2), the increase of alpha-2 serum globulins reproduces experimentally a pathologic condition already reported in chronic nephritic patients(4,5). Alpha-2 globulin increase could be caused, according to Eiselt and Hrabane(4), by adsorption of lipids secondary to hyperlipemia which is common in this disease. However, since it appears in chronic forms of both human and experimental nephritis, the possibility of increased serum hypertensinogen (also an alpha-2 euglobulin) should not be dis-

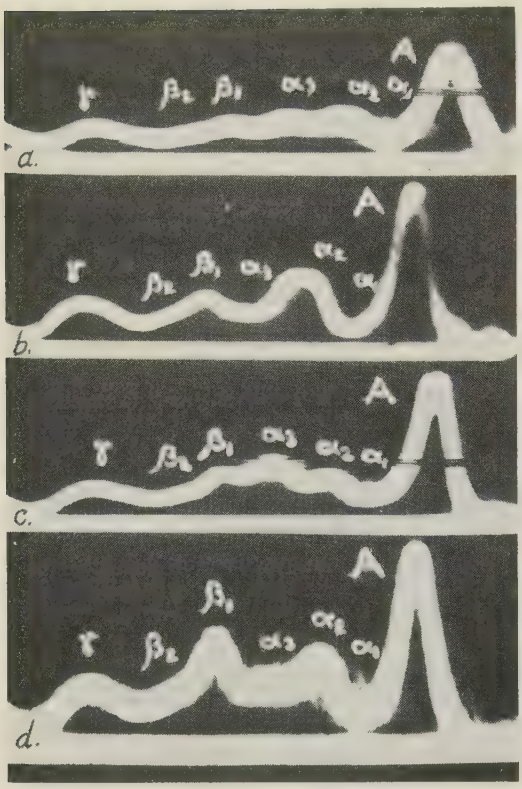

FIG. 3. Alteration of serum electrophoretic pattern during experimental nephritis in rabbits: (a) control; (b) saline-drinking; (c) uninephrectomized; (d) uninephrectomized plus saline.

counted. The conditioning effect of salt, demonstrated in chronic forms of experimental nephritis(1,2), confirmed observations of Barker and Robinson in humans(6).

When nephrotoxic serum was given to animals preconditioned by surgical removal of one kidney, the alpha-3 globulin fraction was significantly increased. The animals exhibited severe nephritis-nephrosis lesions presumably because the residual kidney received the same amount of nephrotoxic antibodies absorbed by 2 kidneys in controls(2). The association between nephritis-nephrosis lesions and increased alpha-3 globulin can be considered another experimental parallel with the disease in humans. Instead of the alpha-3 fraction which does not exist in human serum, the neighboring alpha-2 and beta globulins increased in adult patients with mixed nephritis-nephrosis(5,8), and in children with lipoid nephrosis(5). The alpha-3 globulin found as constant component of rabbit serum in above determinations was not reported in the work of Ellis using paper electrophoresis. The Figures suggest that the intermediary fraction was included in neighboring alpha and beta globulins(7).

Summary. 1. Experimental glomerulonephritis following administration of antirabbit kidney duck serum was induced in normal and preconditioned rabbits. 2. Significant alterations in serum globulins were recorded only in preconditioned animals. a) Rabbits preconditioned with 0.9% saline-drinking water developed clinical symptoms of chronic glomerulonephritis and showed significant increase of alpha-2 serum globulin fraction. b) Animals preconditioned by uninephrectomy showed severe nephritis-nephrosis lesions associated with significant increase of serum globulin fraction situated between alpha-2 and beta globulin (alpha-3 globulin) in the moving boundary electrophoretic patterns.

Angeline Elder performed electrophoretic determinations and is thankfully acknowledged. The authors are also grateful to Dr. Bruno W. Volk, director of Isaac Albert Research Inst. for aid and encouragement.

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Serum Elastase Inhibitor. Levels in Animal and Human Sera, including Selected Disease States.* (24822)

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The elastolytic enzyme of pancreas, elastase, can exert *in vitro* a powerful solubilizing action upon ordinarily highly insoluble scleroprotein elastin, and is the only known mammalian enzyme to have this effect. The role of the enzyme in the body is largely unknown. Animal and human sera possess an inhibitor for the enzyme(1,2,3). In the present study of inhibitor substance we shall present data concerning(1) certain variables involved in measuring the elastase-inhibitor level of serum, (2) localization of the inhibitor among serum fractions separated by starch-block electrophoresis, and (3) representative levels of the inhibitor in the sera of certain animal species, normal values for healthy humans of various age groups, and levels in selected disease states in the human.

Materials and methods. Elastase was prepared as previously described(3). The elastin substrate was made by treating ground defatted human aorta with 0.1 N NaOH at a temperature of 98°C for 45 minutes, according to the method of Lansing *et al.*(4). Unless otherwise stated, the inhibitor test system consisted of 20 mg of substrate, 4 ml of pH 8.0 veronal-acetate buffer containing a total of 0.8 mg of elastase, and 0.15 ml of the serum to be tested. The reagents were rapidly added in the order given. Controls consisted of the same quantity of substrate plus 4.15 ml of buffer containing a total of 0.8 mg of elastase. The tubes were incubated at 37°C for one hour. Percent inhibition was then determined by gravimetric data, using the formula:

$$\% \text{ inhibition} = \frac{\text{No. mg elastin solubilized in control} - \text{No. mg elastin solubilized in test}}{\text{No. mg elastin solubilized in control}} \times 100.$$

Serum fractions were obtained using starch-block electrophoresis with veronal-acetate buffer of pH 8.6. Fractions were eluted from each 0.5 cm of the block, and aliquots analyzed for protein content by Lowry's method (5): Inhibitor levels of representative fractions were then measured both by employing equal volumes of eluate and by employing aliquots adjusted to contain equal protein content. The original electrophoretic curve was then compared by visual inspection with these two types of inhibitor curve (Fig. 2).

Results. Inspection of Fig. 1 clearly indicates that per cent inhibition varies with time. A small portion of this variability might be explained by a mild thermolability of the inhibitor. Serum pre-heated to 37°C for 2 hours retained at least 75% of its inhibitory activity. Heating serum to 45°C for 20 minutes did not destroy its inhibitory effect, but at 55°C for the same time period a marked loss in activity was observed. The actual mechanism of the inhibition and the kinetics of the reaction cannot be assessed from data thus far collected. It may be said, however, that level of serum inhibitor is not influenced by pre-exposure of the serum to an excess of purified elastin.

Fig. 2 indicates that the inhibitor substance is located approximately at the alpha$_1$-globulin/albumin junction of the electrophoretic curve of serum, and is absent from other globulin fractions.

Inhibitor levels in the sera of certain animal species are specified in Table I. Time curves for dog serum showed a pattern qualitatively quite similar to that of Fig. 1 for human serum. For 21 human male sera mean value of elastase-inhibitor under the particular conditions of our experiments was 56.6% with a standard deviation of ± 10.5. Levels in females appeared slightly higher, with a mean value for 8 sera of 65.9%. No significant variation in inhibitor levels with age of the individuals from the newborn period to 68 years was detected. In Table II are presented in-

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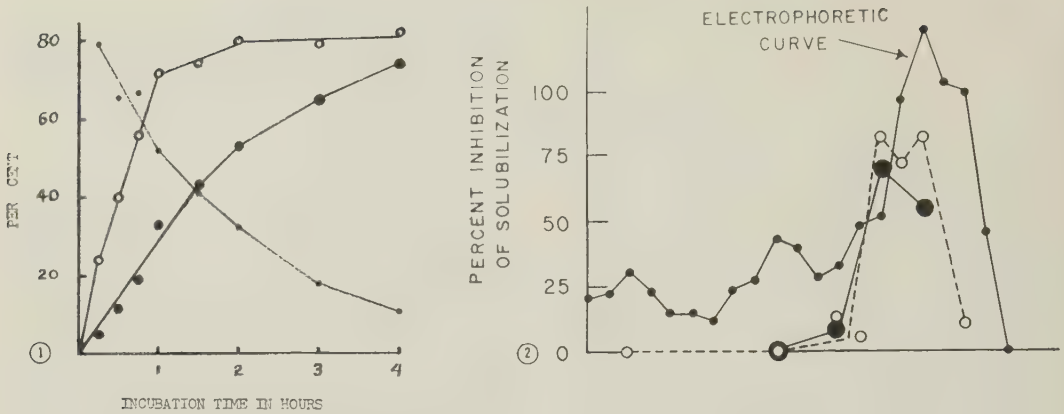

FIG. 1. Effect of time of incubation on measurement of elastase-inhibitor of human serum. For these curves the system comprised 20 mg elastin, 0.5 mg elastase, 4.0 ml buffer, and 0.1 ml of either serum or additional buffer. ○ = % elastin solubilized in control; ● = % elastin solubilized in presence of inhibitor (serum); • = % inhibition.

FIG. 2. Localization of elastase-inhibitor activity in serum fractions obtained by starch-block electrophoresis. One curve (○- - -○) was obtained by testing equal volumes of eluate, another (● ●) by testing aliquots of different volume but of equal protein content.

hibitor levels in certain physiologic or pathologic conditions in the human. These include the third trimester of pregnancy, arteriosclerosis, liver injury (the criterion for selection here being a 4+ cephalin cholesterol flocculation), disseminated lupus erythematosus, dermatomyositis, and severe nephrosis. These diseases were selected as representing either greatly altered protein mtabolism, or conditions in which elastic tissue injury might conceivably be anticipated, with the idea that such changes might be reflected in altered serum elastase-inhibitor levels. Consistently high levels were obtained in pregnancy. There seemed to be a moderate increase in serum elastase-inhibitor in a number of the disease states investigated, but no particular pattern was evident.

Discussion. A comment is in order about the reproducibility of elastase-inhibitor determinations performed at the arbitrary incubation time of one hour. To test this point, 5 separate, single determinations were made over a 3-month interval on aliquots of the same sample of human serum. The values, in the order obtained, were 47.5, 50.9, 49.3, 46.1, and 45.7% inhibition. This seems very satisfactory concordance for a measurement of this type. It is true that the curves for per cent elastin solubilized in control and in presence of inhibitor (as illustrated in Fig. 1) may vary somewhat from day to day. The variation is not marked, however, and the 2 curves vary in a proportional manner. Hence the overall per cent inhibition remains reasonably constant in separate determinations done at

TABLE I. Representative Values for Elastase-Inhibitor in Sera of Normal Adult Animals. Values are given as % inhibition for 3 or more individuals of each species.

Species	% inhibition
Chicken	55.2, 63.7, 67.5
Rabbit	48.7, 53.8, 52.3
Guinea-pig	56.1, 63.0, 52.0
Rat	37.9, 55.1, 57.6, 59.2
Mouse	78.4, 82.0, 85.7, 92.2
Sheep	59.8, 60.9, 79.0
Dog	21.8, 26.6, 36.8
Monkey	40.4, 55.6, 59.0

TABLE II. Levels of Serum Elastase-Inhibitor in Third Trimester Pregnancy, and in Selected Disease States in the Human.

Condition	% inhibition
Pregnancy, third trimester	89.4, 91.7, 94.7, 96.0, 97.8, 98.0, 98.6, 99.0, 99.5
Disseminated L.E.	58.4, 62.4, 71.8, 81.3, 82.6
Dermatomyositis	24.1, 81.3, 81.8, 85.7, 86.4
Arteriosclerosis*	46.9, 51.0, 59.0, 61.5, 71.4, 95.3
Liver damage (4+ ceph. floc.)	35.9,† 69.2,† 79.3,† 88.5,‡ 89.3§
Nephrotic syndrome	69.6, 77.9, 94.2

* All patients less than 60 years old.
† Laennec's cirrhosis.
‡ Infectious hepatitis.
§ Paraproteinemia, type undetermined.

the same time intervals up to and including one hour. At 2 and 4 hours of incubation, however, the determination is not highly reproducible. It should also be remarked that certain adjustments must be made in the test system if different batches of elastase itself are used, since the enzyme preparation, while quite potent, is not pure. This can be done by using stored frozen serum as a standard, in accordance with the values cited above. All numerical data presented in this paper, however, were obtained by use of a single batch of the enzyme. In a number of experiments we found that addition of twice the amount of serum led approximately to a doubling of per cent inhibition, and correspondingly for other amounts. This accords with results published by Banga *et al.*(2) and indicates that per cent inhibition bears a quantitative relation to a serum factor or factors.

Because elastase is known to have potent proteolytic activity against a wide variety of substrates(6), the question arises whether the inhibitor is indeed a specific substance, or whether the inhibition is merely a phenomenon of general protein interference. Evidence seems to favor the former view and may be set forth as follows. In separate experiments we found that casein—which is rapidly and extensively hydrolyzed by elastase—has only a negligible inhibitory effect upon elastolytic activity of the enzyme. At the same time, the serum elastase-inhibitor has no measurable inhibitory effect upon casein-proteolytic activity of the enzyme(3). Secondly the inhibitory activity of serum is located in a specific area of the electrophoretic curve as illustrated in Fig. 2. Thirdly, we found no relation between serum elastase inhibitor activity and total protein content of 7 human serum specimens with total protein values ranging from 3.6 to 7.9 g per 100 ml. Paper electrophoretic protein patterns were also available for these 7 specimens. An attempt was made to correlate per cent elastase inhibition with absolute values for $alpha_1$-globulin and/or albumin in these sera, in view of our results with eluates from starch-block electrophoresis (Fig. 2). No strict correlation obtained, although the 2 highest inhibitor levels were shown by the 2 sera with highest $alpha_1$-globulin fractions, and the lowest inhibitor level by the serum with the lowest $alpha_1$-globulin fraction. While the inhibitor resides in the $alpha_1$-globulin/albumin portion of serum, it is not simply proportional to the absolute quantity of either of these whole fractions.

The level of elastase-inhibitor in normal human serum appeared to be independent of age. Individuals tended to maintain the same inhibitor levels when examined on successive occasions. Inhibitor level did not vary significantly with ingestion of food, including fat-rich meals. Consistently high values were obtained in sera from the third trimester of pregnancy (Table II). This may be of some interest in view of the increased incidence of dissecting aortic aneurysm among those females less than 40 years of age who are pregnant(7). In this age group dissecting aneurysm is particularly associated with elastic tissue lesions. The serum elastase-inhibitor appeared moderately elevated in most instances of all the pathologic states studied. No explanation exists at present for the occational low or very high values obtained (Table II). We were not able to confirm the existence of abnormally low values for elastase-inhibitor in sera of patients with arteriosclerosis, as reported by Balo and Banga(1). Our values for animal sera (Table I) ought not to be taken for more than they are worth, since time curves and other studies were not done except for man and the dog. The animal data are merely meant to suggest that a wide variety of animal sera also contain elastase-inhibitor, and probably in a range broadly comparable to human serum.

Summary. A study of certain variables involved in measuring elastase-inhibitor activity of animal and human serum is presented, with particular emphasis upon time of incubation of the system. The inhibitor is shown to reside at the $alpha_1$-globulin/albumin junction of electrophoretically fractionated serum, but is not itself directly proportional to absolute values of either of these fractions. Inhibitor level in humans is probably independent of age. Mean value for males is about 56.6% under the experimental conditions outlined in

this report. Value for females is somewhat higher. Representative levels for sera of chicken, rabbit, guinea-pig, rat, mouse, sheep, dog, and monkey are given. Inhibitor is greatly elevated in sera of human females during the third trimester of pregnancy. In a number of selected disease states the inhibitor was found usually to be moderately elevated, although no consistent pattern was noted. The inhibitor was not diminished in sera of patients with arteriosclerosis.

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Alpha-2 Lipoprotein in Man and Its Relation to Myocardial Infarction.*† (24823)

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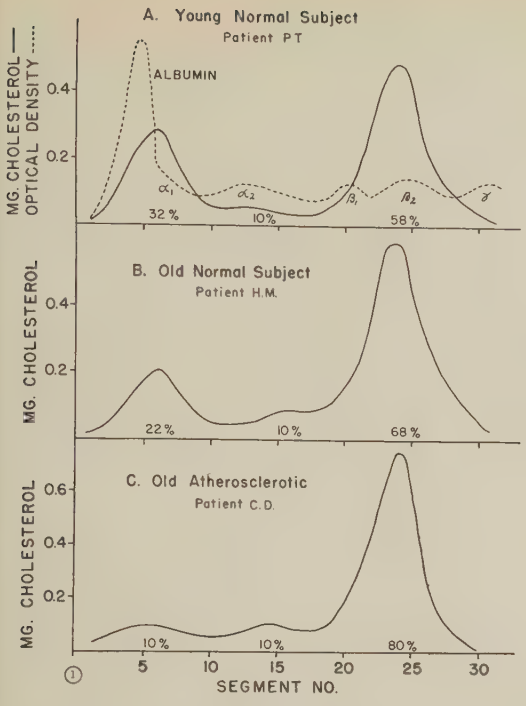
In studies on human serum lipoproteins using starch zone electrophoresis, the lipoproteins, as measured by cholesterol distribution, are readily separated into 3 main fractions (1,2). Fig. 1-A shows a cholesterol distribution curve obtained from a normal young male subject together with corresponding protein distribution curve. In healthy elderly individuals a similar curve is obtained as shown in Fig. 1-B, with a decrease in alpha-1 cholesterol percentage of approximately ⅓ and a corresponding increase in beta cholesterol, with no change in alpha-2 cholesterol percentage. Among elderly individuals with moderate or advanced atherosclerosis, a common type of curve is shown in Fig. 1-C, with a higher percentage of cholesterol in the beta fraction. Similar results have been noted by other workers(3,4,5) using paper electrophoresis in which only alpha and beta fractions are usually reported. In 24% of our male subjects there were lipoprotein curves with increased percentage of cholesterol in the alpha-2 fraction. Recently E. B. Smith(6) using paper electrophoresis noted a component which migrated between alpha and beta fractions (designated pre-beta fraction by this author) which appeared associated with presence of recent myocardial infarction in the patient. Schettler and his coworkers(7) using starch electrophoresis also mentioned that changes in the lipoprotein pattern may occur with myocardial infarction. We were therefore interested in determining the incidence of such diseased states among subjects with increased alpha-2 cholesterol.

Methods. Our subjects were patients of one of the authors (WBK). Lipoprotein determinations were made on selected patients visiting the physician for periodic check-ups. Ages varied from 35 to 83 years, most between 50 and 70. Three hundred male patients, taken in order, were used. The subjects were all of better than average economic status and presumably all had adequate dietary intake which was probably relatively high in fats. All blood samples were obtained in the morning with subjects in basal state and lipoprotein determinations carried out by the method used earlier(2).

Results. Patients were divided into groups on the basis of percentage of cholesterol found in the alpha-2 fraction. Thirty-eight, or

* Supported, in part, by funds from Gerontological Research Fn.

† A portion of this material was presented by senior author before First Pan-Amercan Congress of Gerontology, Sept. 22, 1956, Mexico City.



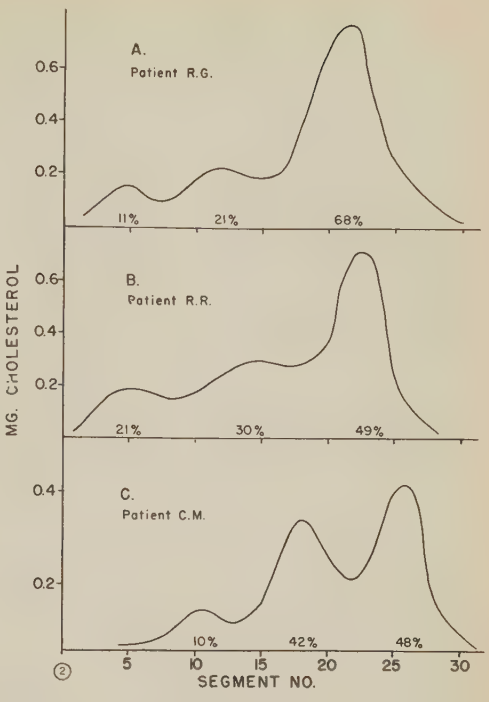


FIG. 1 (left). A. Cholesterol lipoprotein curve and protein curve on a 27-yr-old healthy male subject. B . Cholesterol lipoprotein curve on a 68-yr-old male in good health. C. Cholesterol lipoprotein curve on a 70-yr-old male with advanced atherosclerosis.

FIG. 2 (right). A, B, C. Cholesterol lipoprotein curves on men with elevated alpha-2 fractions and histories of previous myocardial infarctions. Ages of patients are 61, 48 and 52 yr, respectively.

12.6%, had an alpha-2 cholesterol of over 20% (Group I), 34, or 11.3%, had alpha-2 cholesterol of between 16 and 20% inclusive (Group II), and the remaining 228, or 76.0%, had an alpha-2 cholesterol of less than 16% (Group III). After classification on the basis of alpha-2 lipoprotein level, histories were examined for evidence of previous myocardial infarctions or other cardiovascular disease. The results are shown in Table I.

Of those in Group I, 28 had histories of definite myocardial infarcts from 2 months to

TABLE I. Lipoprotein Cholesterol Distribution in Relation to Myocardial Infarction. Average serum cholesterol levels in mg/100 ml and percentage of cholesterol in different fraction. (Figures in parentheses represent range of values. See text for detailed explanation of groupings.)

	No.	Total cholesterol (mg %)	Cholesterol distribution (%) α-1	α-2	β
Group I (avg age 56)					
1. With myocardial infarction	28	306 (205–390)	13 (9–21)	24 (21–49)	62 (41–71)
2. Other disease (see text)	7	375 (305–490)	8 (5–12)	48 (20–69)	44 (25–74)
3. No infarct	3	(165–205)	(15–28)	(21–23)	(50–71)
Group II (avg age 55)					
1. With myocardial infarction	10	304 (225–395)	13 (12–15)	19 (16–20)	68 (65–75)
2. With hypertensive cardiovascular disease	7	280 (230–315)	16 (9–22)	18 (16–20)	66 (63–72)
3. Little detectable cardiac disease	17	225 (195–310)	21 (17–26)	17 (16–20)	62 (57–68)
Group III (Avg age 62)					
"Normal" alpha-2 lipoprotein	228	280 (180–360)	18 (10–25)	12 (8–15)	70 (60–80)

2 years previous, as indicated by clinical evidence and electrocardiographic changes. Typical lipoprotein curves for 3 of these individuals are shown in Fig. 2. Note distinct alpha-2 peak in all instances.

Seven additional patients had very high alpha-2 fractions. All had hyperlipemia, nephrosis, liver disease, or other conditions known to cause a markedly elevated alpha-2 cholesterol(8). Since the elevation due to these causes could completely mask any changes due to possible myocardial infarction, these subjects were not considered further. The 3 remaining patients in Group I showed no positive evidence, clinically or electrocardiographically, of previous myocardial infarction.

Ten of the patients in Group II had a history of previous myocardial infarctions as indicated by clinical symptoms and electrocardiographic changes, 7 more had moderate to severe hypertensive cardiovascular disease, but without any definite evidence of previous infarction, while the remaining one-half had little evidence of cardiovascular disease. Of the 228 remaining patients with normal alpha-2 lipoprotein cholesterol percentage (Group III), only 3 showed any definite clinical or electrocardiographic evidence of infarction in the past.

Discussion. The fact that 28 of 31 patients with more than 20% of cholesterol in the alpha-2 fraction (excluding those with nephrosis, hyperlipemia, etc.) had histories of previous myocardial infarction, is a strong indication of some relationship between lipoprotein distribution and myocardial infarcts. Considering patients listed in Table I (except Group 1-2, all 65 patients with more than 16% of cholesterol in the alpha-2 fraction, 38 have histories of myocardial infarction, 7 have hypertensive cardiovascular disease without definite evidence of myocardial infarct and 20 have little detectable cardiac disease. The difference in alpha-1 cholesterol is also of interest. In 38 patients with infarcts, the average alpha-1 cholesterol was 13%, and in 20 patients without infarcts, the average was 21%. The difference between these averages was significant at the 1% level. In patients with only moderately increased alpha-2 cholesterol, an accompanying lower alpha-1 level is additional indication of a possible previous infarction. If criteria used to divide patients into 2 groups were changed as follows: one group with alpha-2 cholesterol of more than 15% and an accompanying alpha-1 cholesterol numerically at least 3% less (*e.g.*, with alpha-2 cholesterol of say 16% and an alpha-1 cholesterol of 13% or less, or with an alpha-2 cholesterol of say 20% and an alpha-1 cholesterol of 17% or less), and all other patients in a second group, the first group would then contain 38 patients with a history of myocardial infarction and 4 without, while the second group of 255 patients would contain only 3 patients with a history of myocardial infarction. The large differences between these 2 groups would indicate a very definite correlation between abnormal type of lipoprotein curve and history of previous myocardial infarcts. The increased alpha-2 would apparently be the primary determinant and the decreased alpha-1 would be of secondary but adjunctive significance. Of 4 patients in the first group without a definite diagnosis of a myocardial infarct, 3 had moderate to severe hypertensive cardiovascular disease. The fourth patient was a young physician of 35 who had a typically abnormal curve with a cholesterol distribution of 15%, 23% and 62% but who denied any symptoms of even a mild infarction.

When lipoprotein distribution curves were obtained, all subjects with previous myocardial infarcts had made successful recoveries. The data do not tell us whether the abnormal curves existed prior to infarction or how long they had been present. E. B. Smith(6) found that the pre-beta fraction developed within a few days after infarction and disappeared within a few weeks. Because of the difference in technics, comparison with our results is difficult. We have made repeated determinations on some patients with infarctions and in only 2 out of 17 did the distinguishing features of lipoprotein distribution disappear. In one instance the abnormal alpha-2 peak had disappeared when a second determination was made 2 months later. In the second instance the determination was not repeated until 2 years after the initial calculation. With other

patients on whom repeated determinations were made, the character of the curve has remained essentially unchanged over periods as long as 2 years. The time between myocardial infarction and when the initial curve was obtained, also varied from a few months to over a year. The fact that the abnormal features of the lipoprotein curve can disappear might account for the 3 patients (out of 228) with normal curves with a history of previous myocardial infarction. Our observations would therefore indicate that the character of the curves is relatively constant. At present we cannot state whether such curves occur prior to myocardial infarction. Lipoprotein curves were made from 2 elderly patients who died within a few days after a myocardial infarction. The results showed relatively normal curves, suggesting abnormal lipoprotein distribution occurs at some interval after infarction. Our results, however, are based almost entirely on patients who have survived myocardial infarction as compared with those who have not had one.

Summary and conclusions. Data are presented which indicate that an abnormal type of cholesterol lipoprotein curve is associated with a history of recovery from a previous myocardial infarction. The type of curve obtained from a given individual is relatively constant over years. As yet we are unable to say whether the abnormal type of curve develops prior to, or after, initial infarction, or whether degree of abnormality is of any prognostic value. The relationship which exists suggests the need for further studies as to diagnostic and prognostic value of this method in the study of myocardial infarction.

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Fixation of 5-Hydroxytryptamine by Brain Mitochondria.* (24824)

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In investigating some physical and biochemical properties of brain mitochondria, it seemed desirable to know whether any neurohumoral amines present in the brain were attached to mitochondria and involved in their physiology. There is evidence to suggest that histamine(1), nor-adrenalin(2) and acetylcholine(3) are associated with mitochondria elsewhere in the organism; but no comparable study has been made with brain mitochondria. Since recent interest in possible role of 5-hydroxytryptamine (HT)† in brain function, and an extremely sensitive bioassay was available, such study was undertaken with this neurohumoral amine. Our report deals with localization of HT in rat brain mitochondria and its possible relationship to mitochondrial metabolism. A preliminary account of this work has been presented(4).

Methods. Mitochondria were prepared from whole rat brain by methods comparable to those described previously(4), with special precautions to eliminate blood cells and cellu-

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† The following abbreviation were used: HT, 5-hydroxytryptamine; P/O, oxidative phosphorylation. HT was kindly supplied by Dr. R. K. Richards of Abbott Labs., Chicago.

TABLE I. Distribution of HT in Cellular Fractions of Whole Rat Brain. Values are expressed in terms of μg/HT/g wet wt of whole brain and are an average of 3 experiments agreeing within 6%.

Cellular fraction	Amt HT, μg/g	% total
Supernatant	.027	12
Nuclear-debris	.052	22
Mitochondria	.149	63
Microsomes	.002	0
Whole brain	.235	100
% recovery		98

lar debris. A 5% homogenate of brain in 0.25 M sucrose, containing 10^{-4} M sodium versenate was subjected to 3 successive centrifugations at 1200 x g, to remove nuclei, erythrocytes, and cellular debris. The carefully decanted supernatant was then centrifuged at 10,000 x g for 20 minutes, to yield the mitochondria fraction. The mitochondrial residue was washed once by homogenizing in 0.25 M sucrose-versene and centrifuging at 12,000 x g for 20 minutes. Sedimentation at 75,000 x g then yielded the "microsome" residue and "supernatant" fraction. All operations were performed at 0°. For a given experiment, mitochondria were prepared from 4 rat brains and usually divided into 8 parts. Each fraction was then incubated with a particular drug in ice bath 30 minutes. After centrifugation at 15,000 x g and washed once in 10 ml of isotonic sucrose-versene, the mitochondria were treated with 20 volumes of acetone to extract HT. The residue was again extracted with 90% acetone, and extracts combined and dried *in vacuo*. After the dried residue was suspended in 2 ml distilled water, the lipids were removed by extracting twice with 10 ml of petroleum ether. The water layer was then dried *in vacuo* and ready for bioassay. Estimation of concentration of HT was made by the method of Amin *et al.*(6), using isolated rat uterus at 29° and compared to standard solution of 5-hydroxytryptamine creatine sulfate. Some samples were assayed on the isolated rat uterus in superfusion(7) for greater sensitivity. Measurement of P/O† of brain mitochondria was described previously(5). All agents were homogenized with the mitochondrial preparations and preincubated at 0° 20 minutes before determining P/O. Mitochondrial preparations were tested for P/O before use, and those not of optimal activity were discarded.

TABLE II. Effect of Reserpine and Other Agents on HT (μg) Content of Mitochondria from 1 g Rat Brain. Mitochondria were incubated with reserpine for 20 min. at 0°, then centrifuged at 15,000 × g and washed once. HT was then extracted from supernatant and mitochondria.

	Supernatant	Mitochondria
Control	.027	.200
	.060	.200
	.010	.120
	.035	.135
Reserpine	.150	.020
	.075	.015
	.125	.020
	.135	.045
Dist. water	.080	.100
Detergent*	.125	.010

* .02% sodium lauryl sulfate.

Results. Results on distribution of HT among various cytoplasmic fractions of rat brain are presented in Table I. The mitochondrial fraction contained 63% of total HT in rat brain, while the nuclear-debris fraction contained 22% and supernatant 12%.

TABLE III. Release and Binding of HT in Rat Brain Mitochondria. Concentration of reserpine and HT was 10^{-6} M. Results are average of 2 experiments agreeing within 10%.

	Original HT	Net change in HT	
	(μg/g)		% change
Control	.15		
After reserpine	.02		–87
After HT	.40	.25	255
Reserpine, then HT	.30	.28	300

Reserpine at concentration of 10^{-6} M almost completely released HT from mitochondria (Table II). Suspending mitochondria in distilled water for 15 minutes caused only a partial release of the bound HT, while exposure to .02% solution of a detergent resulted in almost complete loss of mitochondrial HT (Table II).

Freshly prepared mitochondria exhibited the capacity to bind over twice their original content of HT (Table III). After depletion of HT by pre-treatment with reserpine, mitochondria absorbed twice the original amount of HT; however, this value of 0.30 μg/g was 0.10 μg less than total amount of HT absorbed without treatment with reserpine.

Various agents other than reserpine were

TABLE IV. Effect of Various Agents on HT Binding of Rat Brain Mitochondria. Values are expressed in terms of μg/HT/g wet wt of whole rat brain, and represent avg of 2 experiments with each agent.

Agent	Conc., M	Control	After agent	After agent + HT	Agent + HT after reserpine depletion
LSD-25	10^{-5}	.09	.09	.18	.10
Reserpine	10^{-6}	.09	.003	.18	
Phenyl ether	"	.15	.14	.30	.02
Dibenamine	10^{-5}	.09	.09		.13
DNP	"	.15	.16	.40	.38
None		.15		.42	.30

tested for possible effect on binding and release of HT in rat brain mitochondria (Table IV). LSD-25, phenyl ether, and dinitrophenol were without effect on mitochondrial concentration of HT. Dinitrophenol did not affect binding of HT, while LSD, reserpine, and phenyl ether produced some inhibition. After pre-treatment with reserpine, binding of HT by mitochondria was almost completely inhibited by phenyl ether, and inhibited over 50% by dibenamine, while LSD and dinitrophenol produced no change.

The various agents were tested for effect on oxidative phosphorylation of rat brain mitochondria (Table V). SKF 501, phenyl ether, and dibenamine all produced about 50% inhibition at concentrations slightly over 10^{-5} M. LSD-25‡ and reserpine caused some inhibition at 5 x 10^{-5} M, while HT produced 26% inhibition at 10^{-4} M. The more potent inhibitors compared in effectiveness with dinitrophenol.

Discussion. Binding of HT by rat brain mitochondria is not unexpected, since other neurohumoral amines, such as adrenaline(1), histamine(2), and acetylcholine(3) are likewise present. Although the role of various neurohumoral amines in the central nervous system is uncertain, their effects on peripheral nervous system and smooth muscle have been extensively studied(8,9,10).

That dibenamine, phenyl ether, and reserpine interfere with HT binding by mitochondria is interesting, since some of these agents block contraction of smooth muscle by HT (11). All 3 agents will inhibit oxidative phosphorylation at concentrations of 10^{-5} M; but since substances exist which do not affect phosphorylation but block HT, and other inhibitors of oxidative phosphorylation (such as dinitrophenol) do not block HT, there seems to be no distinct relationship between "uncoupling action" and HT blockade. On the other hand, the mechanism of inhibition of phosphorylation by substances such as reserpine, dibenamine, and phenyl ether is different from that of dinitrophenol, which fails to block the action of HT(12). It is conceivable, therefore, that some overlapping exists between the phosphorylative portion of mitochondria and receptor sites for HT. Further evidence for a possible relationship between

TABLE V. Inhibition of Oxidative Phosphorylation (P/O) of Rat Brain Mitochondria by "Diphenyl" Agents Affecting HT Contraction of Smooth Muscle. Homogenation of agent with mitochondria and pre-incubation at 0° for 20 min. was necessary for maximal inhibition. Results are avg of 3 experiments agreeing within 7%.

Agent	Conc., M	△O, μatoms	△P, μmoles	P/O	% inhib. P/O
Control		6.1	16.6	2.7	
Reserpine	2 × 10^{-5}	5.5	12.8	2.3	12
SKF-501	3 "	3.8	4.6	1.2	56
LSD-25	5 "	5.8	12.0	2.2	18
Phenyl ether	5 "	4.0	4.1	1.0	67
Dibenamine	2 "	5.2	9.1	1.7	37
Dinitrophenol	5 "	6.1	9.0	1.5	45
HT	10^{-4}	5.8	2.9	2.0	26

‡ Studies with LSD-25 were performed with crystalline form of LSD-25, generously supplied by Sandoz Pharmaceutical Co. With respect to oxidative phosphorylation of brain mitochondria, the solution preparation supplied in 100 μg amounts in sealed vials by Sandoz proved 50 times more potent an inhibitor. Dr. Julia Apter has personally communicated that entirely different results are obtained in electroretinogram studies employing crystalline and solution preparations of LSD-25.

phosphorylation and binding of HT is that concentration of HT in platelets is partly dependent upon the ATP present(13).

That the major fraction of HT is bound to the mitochondria appears to conflict with the report of Hughes *et al.*(14) that the amine is not specifically bound to any cytoplasmic fraction. Whether this difference can be due to the fact that these authors used rabbit instead of rat brain remains to be determined. Since the slightest mistreatment of mitochondria during isolation alters both their phosphorylative efficiency and HT binding, precautions were necessary to obtain good results. Our experience has been that oxidative phosphorylation of rabbit brain mitochondria has appreciably lower specific activity than rat brain, in addition to being more labile. Baker (15) reported that most of the HT of the gastric mucosa is present in the mitochondrial fraction, and that HT is released upon exposure to a hypotonic medium.

The following can be stated with regard to the mitochondrial "receptor site" and the nature of the binding of HT. From the point of view of chemical constitution, the similarity of action to reserpine of such agents as SKF 501, dibenamine, and phenyl ether, is not unexpected(12). It had been previously reported that oxidative phosphorylation could also be restored after brain mitochondria were washed free of reserpine(12). In isolated mitochondria, reserpine does not appear to produce alteration in "receptor sites," whereas *in vivo,* capacity of brain to bind HT was inhibited long after elimination of administered reserpine(16). As far as isolated mitochondria are concerned, the only agent studied which brought about irreversible change in either HT binding or oxidative phosphorylation(12) was SKF 501. Although the nature of the binding is obscure, it appears that either mitochondrial structure or some peculiar constituent is in some way involved.

Summary. Distribution of 5-hydroxytryptamine (HT) in various cytoplasmic fractions of rat brain has been determined, and the neurohumor was confined almost exclusively to the mitochondrial fraction. Numerous inhibitors of oxidative phosphorylation were tested for ability to release HT from mitochondria or to prevent binding. Reserpine completely depleted mitochondria of HT, but did not alter their ability to reabsorb HT, while phenyl ether and LSD partly prevented mitochondrial fixation of HT.

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Plasma Aldolase and Glutamic-Oxaloacetic Transaminase Activities in Inherited Muscular Dystrophy of Domestic Chicken.* (24825)

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Increased serum aldolase(1,2) and glutamic-oxaloacetic transaminase(3) (SGO-T) activities have been observed in human progressive muscular dystrophy. Elevations in serum aldolase activity have also been reported in hepatitis(4,5), prostatic carcinoma(6,7), and acute myocardial infarction(8) of man. Only recently, enzyme studies in mice which show a hereditary muscular dystrophy, revealed increases in serum aldolase activity (9). These studies indicated an unchanged muscle aldolase activity in reference to non-collagen protein nitrogen of the tissue. Succinoxidase activity of dystrophic muscle tissue was not significantly affected, while cytochrome oxidase and cathepsin activities were markedly increased(9). In human patients with muscular dystrophy, it has been reported that serum GO-T activity increases while muscle GO-T activity, in reference to non-collagen protein nitrogen, remains unchanged(10). Weinstock *et al.*(9) have tentatively concluded that in human clinical muscular dystrophy or atrophy from neurotomy, glycolytic enzyme activity generally decreases and respiratory enzyme activity is not affected. In contrast to this, respiratory enzyme activity tends to increase and glycolytic enzyme activity remains relatively unchanged in muscular dystrophy induced by Vit. E deficiency or the hereditary type observed in inbred mice. Myocardial infarction(11) and hepatic necrosis in dogs(12) has recently been found associated with increased SGO-T activity in serum. It has also been observed that in bovine(13) and ovine(13,14) white muscle disease, a marked increase in SGO-T activity was present. Kuttler and Marble(14) observed increases in SGO-T activity in experimental white muscle disease of sheep as early as 12 days prior to development of clinical signs of the disease. They observed that levels of SGO-T roughly paralleled severity of clinical symptoms. Normal values concerning plasma and tissue activity of GO-T in chickens and other domestic animals have been reported by Cornelius *et al.* (12). Asmundson and Julian(15) described an inherited muscular abnormality in New Hampshire chickens. Gross and microscopic pathology of muscles appeared comparable to some of the muscular dystrophies of man. They reported that the affected birds exhibited wider breasts and shorter bones than normal individuals and were homozygous for autosomal recessive gene. Continuing with these investigations in chickens, the present study was undertaken to determine the following: 1. Plasma aldolase and GO-T activities in normal homozygous and heterozygous non-dystrophic male and female New Hampshire chickens; 2. Plasma aldolase and GO-T activities during clinical onset of inherited muscular dystrophy in New Hampshire chickens of strain 3 of the Univ. of California flock; and 3. GO-T activities in tissues of normal and strain 3 dystrophic New Hampshire chickens.

Materials and methods. Individual heparinized blood samples were collected by venipuncture of right jugular vein and centrifuged soon after bleeding. Plasma GO-T activity was measured by the method of Cabaud *et al.* (16). Tissue glutamic oxaloacetic transaminase activity was determined by modification of procedure of Tonhazy *et al.*(17). Samples were incubated 20 minutes at 25°C rather than at 10 minutes at 37°C as in original procedure. Accordingly, both plasma and tissue samples were incubated similarly. The technic for assaying glutamic-oxaloacetic transaminase activity is based upon transfer of the alpha-amino group of aspartic acid to alpha-ketoglutaric acid. The oxaloacetate which results from transamination is converted to pyruvic acid by aniline citrate. A pyruvate-dinitrophenylhydrazone is prepared,

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TABLE I. Mean Plasma Aldolase Activities, as Expressed in "Dihydroxyacetone" Units, in Dystrophic and Normal Birds of Both Sexes Bled at 4 Successive Ages.

	Dystrophic birds				Normal birds	
	Group A		Group N*			
Age (days)	♂	♀	♂	♀	♂	♀
24	4.776 (5)†	1.807 (5)	.980 (3)	1.325 (7)	.955 (5)	.957 (5)
34	5.636 "	4.198 "	2.118 "	3.334 "	.427 "	.646 "
42	2.844 "	1.730 "	2.477 "	3.350 (8)	.741 "	.678 "
50	3.581 "	2.014 "	2.328 "	2.799 (7)	.728 "	.789 "
Avg	4.209	2.437	1.976	2.702	.713	.768

* This group appeared clinically normal between 23-26 days of age.
† Figures in parentheses refer to No. of samples in mean.

extracted with toluene, and measured colorimetrically at 490 mμ. Enzymatic activity is expressed as μg of pyruvic acid liberated in 20 minutes at 25°C/ml of serum or mg of dry tissue. Plasma aldolase was determined by standardization with dihydroxyacetone as recommended by Friedman and Lapan(18). Aldolase liberates both glyceraldehyde-3-phosphate and dihydroxyacetone phosphate from fructose-1, 6-diphosphate. Both trioses exhibit similar color development in a 60 minute period(19). Since both dihydroxyacetone and glyceraldehyde produce identical chromogenic derivates (methylglyoxal-2, 4-dinitrophenylosazone and pyruvic-2, 4-dinitrophenylhydrazone), either triose may be used as secondary standard in place of the more complex analysis for alkali-labile phosphate previously used. In our study, aldolase activity is expressed in terms of "dihydroxyacetone units" in which 1 unit equals amount of color developed by 0.01 mg dihydroxyacetone under the experimental conditions. All data were subjected to statistical analysis by means of analysis of variance. Transformations were made of aldolase data to correct for heterogeneity of variance(20).

Results. Plasma aldolase activities: A summary of results of individual aldolase determinations in plasma from both normal and dystrophic chickens is presented in Table I. Group A consisted of birds which exhibited clinical signs of muscular dystrophy at onset of study, while birds in Group N were free from abnormality at that time. All birds appearing clinically normal exhibited similar normal aldolase activities. Group N, however, showed muscular abnormality at second sampling, associated with increased serum aldolase activity. The data indicate that a pronounced elevation of aldolase activity occurs in all birds exhibiting dystrophy. Group N exhibited no aldolase elevations 7 days prior to occurrence of clinical symptoms of dystrophy. Elevation in activity of this enzyme in plasma in dystrophic birds may represent a change in cellular permeability and/or necrosis. Detailed statistical analyses of variances indicated that in dystrophic birds, age had a highly significant effect, $P<0.01$ (Table I).

Furthermore, interaction between groups and ages, and groups and sexes in dystrophic birds was also significant at same level of probability. This would indicate, therefore, that age and sex of birds as well as presence of muscular abnormality influenced the level of aldolase activity in plasma.

Additional data were obtained from 24 dystrophic and 31 normal birds varying in age from 64 to 365 days. Considering these birds and those reported in Table I, aldolase activities were 2.620 and .652 "dihydroxyacetone" units respectively. Differences due to age were significant with a reduction in aldolase activity in birds over 7 weeks old. It was of special interest to note that when data from older birds were examined by analysis of variance, the effects of sex showed no statistical significance. Considering all aldolase data, the major factors affecting plasma activity were the phenotype and age of bird. The data showed that effect of sex was a factor during early growth of birds but not after birds had passed the age of 7 weeks.

Plasma GO-T activities. Mean plasma GO-T activities in normal and dystrophic birds are presented in Table II. Dystrophic

birds exhibited elevated serum GO-T activities. This difference between dystrophic and normal birds was highly significant ($P<0.01$) but no source of variation, main effect or interaction, was significant except in the case of sex difference in the year-old group of dystrophic birds. Furthermore, analysis of data for each of the four 365-day-old groups, normal and dystrophic (Table II) showed sex genotype interaction, significant at the 5% level of probability. It should be noted that fewer determinations of plasma GO-T activity were performed on samples from young birds than in aldolase activity determinations. Had more ages been represented in the plasma GO-T series, an effect of age and/or sex might have been found. This possibility is substantiated by the observation that correlation between plasma aldolase and GO-T activities was highly significant ($r = 0.69$). This calculation was based upon data from 39 samples in which both aldolase and GO-T activities were determined. Both sexes and groups of birds at 3 different ages were represented.

Tissue GO-T activities. GO-T activities in various tissues of mature birds are presented in Table III. In nearly all tissues analyzed, GO-T activity/mg dry tissue was higher in dystrophic birds. These data show considerable variation in activity of this enzyme among tissues. Statistical analysis revealed that variations among tissues, as well as differences between normal and dystrophic birds were highly significant ($P = <.01$). This does not mean that GO-T activity was higher in all tissues of dystrophic birds but only in the majority of tissues. Higher activities in these tissues were not just a chance occurrence. Much more data are needed to obtain statistically valid inferences for each tissue of dystrophic and normal birds. No other source of variation, including comparison of parenchymous and muscular tissues, significantly influenced this variability. Muscle GO-T activity in human dystrophic muscles was shown to be unchanged when referred to non-collagen protein tissue nitrogen or lower than in normal muscles when related to basis of wet or dry tissue(10) as in our investigation. It was of special interest that muscle GO-T activity/mg dry tissue of dystrophic chickens was in comparison significantly elevated. Since nearly all tissues of dystrophic birds revealed elevated GO-T activities in this investigation, interpretation concerning increased or decreased transaminase activity in dystrophic muscle should be viewed with uncertainty. Relationship of tissue transaminase activity in chickens to protein anabolism and catabolism as reported by Beaton *et al.*(21) in rats merits further investigation.

TABLE II. Mean Plasma GO-T Activities in Dystrophic and Normal Birds of Both Sexes at Various Ages (Pyruvate Liberated/ml Plasma).

Age, days	Dystrophic birds ♂	Dystrophic birds ♀	Normal birds ♂	Normal birds ♀
28	648.7 (6)*	649.1 (6)	226.7(16)	238.2(17)
50	542.0 (5)†	295.5 (5)†	170.0 (5)†	233.6 (5)†
87	837.8(13)	771.0(12)	169.5 (5)†	263.7 (4)†
189	443.9 (5)†	260.0 (5)†		
365	1475.7(10)	819.3(15)	346.4(14)	299.9(12)
Avg	789.6	559.0	228.2	258.9

* Figures in parentheses refer to No. of samples in mean.

† Both plasma GO-T and aldolase activities determined in these samples.

TABLE III. Mean GO-T Activities in Tissues of 12- to 18-Month-Old Dystrophic and Normal Chickens (Pyruvate Liberated/ml Plasma).

Tissue	Dystrophic ♂ (2)*	Dystrophic ♀ (4)	Normal ♂ (3)	Normal ♀ (4)
Superficial pectoral	134	45	89	71
Deep pectoral	114	62	80	76
Biceps	83	123	90	91
Gastrocnemius	228	230	138	103
Gizzard	184	159	129	127
Heart	547	594	453	486
Liver	178	321	181	205
Kidney	243	307	202	228
Spleen	97	89	78	68
Brain	144	234	223	203

* No. of birds in parentheses.

Summary. New Hampshire chickens with onset of inherited muscular dystrophy exhibited elevated plasma aldolase and glutamic oxaloacetic-transaminase (GO-T) activities. Age and sex of birds influenced plasma aldolase activity in both normal and dystrophic groups. GO-T activities varied in different tissues and were greater in a majority of tissues examined from dystrophic birds when compared to normal birds.

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Electrolyte Alterations in Human Plasma and Erythrocytes Associated With Chronic Alcoholism.* (24826)

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Few of the reports in the literature dealing with alcohol intoxication and alcoholism as a medical problem are concerned with its influence on water and electrolyte metabolism. Investigations have been confined to studies of response of normal individuals imbibing alcohol under control conditions, in specific amounts for short periods. Nicholson and Taylor(1) in one of the earliest reports showed that, despite a water diuresis after alcohol administration, Na, K and Cl ions were retained. Only 50% of the retained K could be accounted for in the extracellular fluid of these individuals. Subsequent studies(2,3) have largely complemented the earlier work except that the mode of action of alcohol in production of diuresis has been more accurately delineated(3,4). More recently reports have appeared concerning effects of alcohol on salt and water metabolism in the dog(5,6). Apparently the nature of response to alcohol in this animal is dose-linked. The present report concerns plasma and erythrocyte alterations in 64 human *chronic* alcoholics associated with malnutrition and generalized debilitation.

Methods. The 64 subjects (56 male, 8 female) were chronic alcoholic patients at the Alcoholic Rehabilitation Center. Their ages ranged from 25-67 years with a mean age of 44. All were in poor physical condition with approximately one-half having previous delirium tremens. Most showed signs of poor nutritional background. Ninety % had been drinking heavily for at least 10 years; most had a history of 20 years of alcoholism. While it was difficult to obtain reliable information from the patients, we were reasonably certain that the patients had been continuously drinking from 3 days to 12 months before admission. Beverages consumed consisted of one

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TABLE I. Plasma and Red Blood Cell Electrolyte Values in Normal Medical Students and in Large Group of Chronic Alcoholics.

	Plasma				Erythrocyte			
	Na	K	Cl	Protein,	Na	K	Cl	Hematocrit,
	meq/l			g %	meq/l			%
Normal values								
No. of patients	28	29	27	27	29	29	29	29
Range	138.8–148.8	3.5–5.1	96–126	6.6–7.8	10.3–15.5	93.8–105.0	44–78	43.3–51.0
$\bar{X}$	144.6	4.2	118.8	7.2	12.0	98.6	55.4	47.0
S. D.	± 2.6	± .4	± 8.9	± .3	± 1.2	± 3.7	± 7.7	± 3.9
Chronic alcoholics								
No. of patients	61	61	61	64	62	62	59	60
Range	124.7–151.8	2.5–5.2	76–132	4.9–8.4	9.4–27.8	87.5–108.8	30–74	28.5–50.0
$\bar{X}$	141.8	4.0	102.4	6.8	13.0	101.6	49.4	42.0
S. D.	± 4.2	± .6	± 12.3	± .4	± 3.7	± 4.7	± 8.5	± 4.8
"P"	<.001	<.02	<.001	<.005	<.1	<.005	<.01	<.001

$\bar{X}$ = Arithmetic mean. S. D. = Stand. dev. P = Level of significance.

or some combination of rum, wine, bourbon, gin, beer, vodka, etc. Medical aspects and treatment of this group will be presented later. A 10 ml blood sample was taken on the first day in the Center and before therapy had been administered. This sample was heparinized and centrifuged at 20,000 G in a Servall Super-centrifuge. Plasma and red blood cell Na and K were determined utilizing a Perkin-Elmer Flame Photometer. Chlorides were analyzed chemically by an electrometric modification of the Volhard method(7). Plasma protein was determined by the $CuSO_4$ falling drop method, and hematocrit by the microcapillary technic. Normal values were obtained on 29 normal medical students, and statistical analysis between the 2 groups was made on the basis of unpaired sample data with the "null" hypothesis being rejected at the 5% level.

Results. Experimental results are presented in Table I. Plasma Na, K, Cl and protein concentrations are all significantly reduced in chronic alcoholics as compared to normal medical students. Mean hematocrit value in the alcoholics (42%) is significantly lower ($P<0.001$) than the mean value seen in the controls (47%). Erythrocyte Na remained normal in chronic alcoholic patients, whereas cell K was elevated from a control level of 98.6 to 101.6 meq/l ($P<0.005$) in chronic alcoholics. Red blood cell Cl in alcoholics was reduced an average of 6 meq/l from control values.

Discussion. The long term alcoholic does not usually exhibit the electrolyte alterations which have been reported in cases of acute intoxication(1,2,3,5,6). Nicholson and Taylor (1), utilizing balance studies, showed that retention of K, Na and Cl occurred in short-term observations on medical students given alcohol over an 8 hour period. In contrast, chronic alcoholics, who had been consuming alcoholic beverages excessively for weeks or months prior to study, showed significant reductions in plasma levels of these ions. Patient reports of vomiting over extended periods may account for the hypochloremia seen in these alcoholics. Indeed, the significantly lowered erythrocyte Cl encountered suggests hypochloremia of long duration. Concomitant loss of Na in the vomitus, plus possible compensatory loss of base bicarbonate in the urine, might explain the observed plasma Na reductions. While the reduction in plasma K concentration was statistically significant, very little physiological importance is credited to an average decrease of 0.2 meq/l from control values. However, this study gives little insight into possible alteration in total extracellular K. It is conceivable that in the face of probable reduced extracellular fluid volume (from diuresis and vomiting), some degree of extracellular K depletion occurred which is not readily revealed by observing plasma concentration of this ion. Certainly vomitus can contain a high K concentration

which may result in an inordinate loss of this cation.

It is well known that chronic alcoholism is associated with liver damage. Alcoholic addiction combined with the poor nutritional status of the alcoholic engenders fatty infiltration of the liver with subsequent irreversible hepatic fibrosis. Since the liver is commonly thought to be the site of plasma protein production, and in view of the great probability of serious liver damage in alcoholics, it is not surprising that markedly reduced plasma protein concentrations were encountered in these patients.

The signicant increment in erythrocyte K in the chronic alcoholic agrees with Nicholson and Taylor's conclusion(1) " . . . potassium must be retained in the cells." Their studies, however, were of short duration and involved a relatively limited alcohol consumption, whereas the current experiments involved the long-term, intemperate use of alcohol. Nevertheless, calculations assuming an extracellular fluid volume of 20% of body weight and using average weight of the alcoholic patients (71.6 kg) indicate that an average of 3 meq of extracellular K is lost. Erythrocyte K gain, based on an assumed red blood cell volume of 4% of the body weight, equals 8 meq. From such approximations it is clear that K is either being retained in the chronic alcoholic, or that some marked redistribution of K, associated with altered cell membrane permeability, has occurred.

Bianco and Jolliffe(8) have pointed out that quantitative anemia occurred in 61% of 184 cases of alcoholic addiction, which is not surprising in view of possible complications (stomatitis, pellagra, cirrhosis etc.). In this study average reduction in hematocrit from the control equaled 5% in the alcoholics. While this value was not seriously low (42%), it constitutes a statistically significant departure from the control.

Summary. Plasma and erythrocyte Na, K and Cl have been determined in 64 long-term chronic alcoholics before administration of therapy. Plasma Na, K, Cl and red blood cell Cl were significantly reduced below control values. Erythrocyte K was significantly elevated, while Na remained unchanged. Plasma protein concentration and hematocrit were significantly reduced from control values.

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Observations on Vitamin C Activity of D-Ascorbic Acid.* (24827)

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Previous studies have shown marked differences in retention of D-ascorbic acid-1-C^{14} and L-ascorbic acid-1-C^{14} when administered in small intraperitoneal doses to normal and Vit. C-deficient guinea pigs (1). D-Ascorbic acid is rapidly metabolized and excreted; most of the compound disappears from the body within 12 hours after the dose. In contrast, L-ascorbic acid is

* The authors are grateful to Carole Evans for synthesis of D-ascorbic acid and to Constance Glasgow for assistance in bioassay experiments.

present in the guinea pig for a considerable period of time, disappearing with an average half-life of 3 to 4 days(2). The observation that D-ascorbic acid is retained in guinea pigs to a considerably lesser extent than L-ascorbic acid suggests a possible explanation for the lack of Vit. C activity reported for the D-isomer(3,4). To test this hypothesis, bioassay experiments were carried out in guinea pigs under conditions in which essentially comparable tissue concentrations of D-ascorbic acid and L-ascorbic acid were maintained.

Methods and materials. A modified curative bioassay for Vit. C activity(5) was employed in 2 experiments. *Bioassay 1.* Fifty-four male guinea pigs weighing from 150 to 210 g were given a scorbutogenic diet. On the fifteenth day of the experiment the animals were treated daily for 10 days as follows: One-third received 12 mg of D-ascorbic acid, one-third received 2 mg of L-ascorbic acid and the remaining served as negative controls. *Bioassay 2.* Twenty-three guinea pigs, weighing from 250 to 350 g were placed on the scorbutogenic diet and after 18 days they were treated daily for 8 days as follows: Eight received 4 mg of D-ascorbic acid, 8 received 2 mg of L-ascorbic acid and 7 served as negative controls. In the case of both bioassay experiments, the daily dose of each compound was divided into 2 equal doses and administered by intraperitoneal injection at 12-hour intervals. Negative control animals received injections of saline at the same time intervals. The guinea pigs were weighed daily. At time of sacrifice they were examined for hemorrhages, particularly in the region of the knee joints. The lower jaws were immediately fixed in Lillie's aqueous neutral calcium acetate formalin solution(6) for 24 hrs, decalcified in 5% formic acid, dehydrated, embedded in paraffin and sectioned mesio-distally at 6 μ. Sections were stained with the Rinehart method for acid mucopolysaccharides(7), the alloxan-Schiff method for proteins(8), and Lillie's periodic acid-Schiff method for carbohydrates(6). The Vit. C free diet† was prepared as described by Woodruff, *et al.*(9). D-Ascorbic acid was synthesized by addition of cyanide to D-xylosone(10).

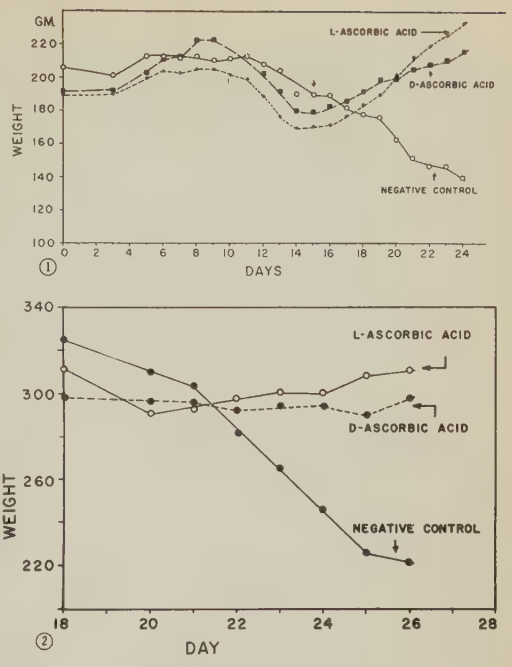


FIG. 1. Wt changes in guinea pig from each of the 3 groups in Bioassay I.

FIG. 2. Wt changes in guinea pig from each of the 3 groups in Bioassay II.

Results. The pattern of weight change in a representative animal from each group in Bioassay I is given in Fig. 1. The animals initially gained weight until about the eleventh day on diet when their weight declined. On the fifteenth day the animals were treated as described in Methods Section. The animals receiving D- and L-ascorbic acid showed comparable weight gains from the fifteenth to twenty-fifth day, averaging 30 g for each group. The guinea pigs treated with D-ascorbic acid had normal appetites and could not be distinguished in gross appearance from those receiving L-ascorbic acid. However, negative control animals all showed a marked loss in weight and had typical scorbutic appearance‡ (5). Out of 18 guinea pigs in the negative control group, only 4 survived at end of experiment, compared to 17 and 16 animals in

† Obtained from Nutritional Biochemicals Co., Cleveland, O.

‡ Sections of liver stained by periodic acid-Schiff method showed that animals in D- and L-ascorbic acid group had normal glycogen content whereas no glycogen was detected in the negative control group.

PLATE I. Photomicrographs of teeth.

A. Apical portion of a molar root from a normal guinea pig. D, dentin; P, pulp; E, enamel; O, odontoblasts. Rinehart stain. × 73.

B. Apical portion of a molar root from a normal guinea pig. Alloxan-Schiff stain. × 73.

C. Apical portion of a molar root from a guinea pig in negative control group. Rinehart stain. Note strong reactivity of stain for acid mucopolysaccharides in the abnormal osteodentin and connective tissues generally. × 73.

D. Apical portion of a molar root from a guinea pig in negative control group. Alloxan-Schiff stain. Note lack of reactivity of stain for proteins in dentin. × 73.

D-ascorbic acid and L-ascorbic acid groups, respectively. Despite ability of D-ascorbic acid to produce a comparable weight gain and survival rate as L-ascorbic acid, upon autopsy the animals receiving D-ascorbic acid showed hemorrhages about the joints similar to those observed in the negative control group. No hemorrhages were detected in the animals receiving L-ascorbic acid.

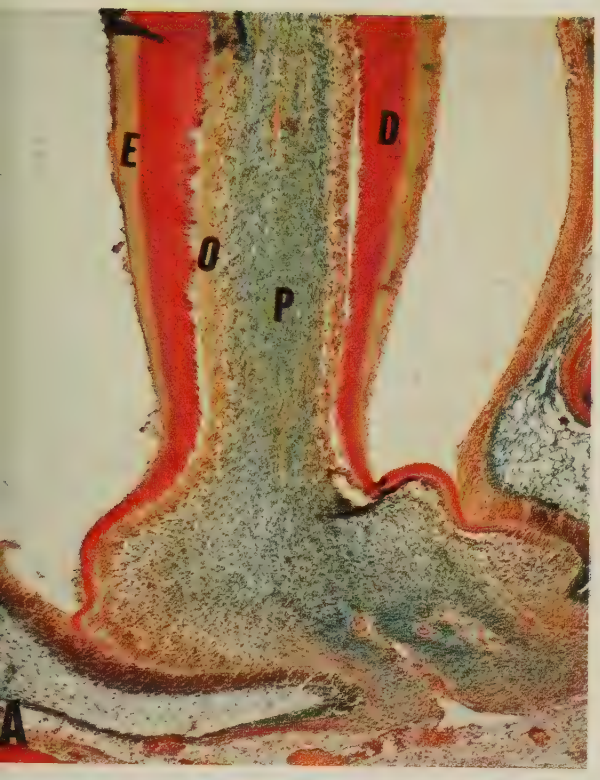
The pattern of weight change in an animal from each group in Bioassay II is given in Fig. 2. The results indicate that even the relatively small dose of D-ascorbic acid employed in this experiment was sufficient to maintain the weight of guinea pigs. In contrast, negative control animals all showed marked loss of weight. Seven out of 8 guinea pigs in each of the D- and L-ascorbic acid groups survived at end of the experiment, compared to 2 out of 7 in the negative control group. Upon autopsy, animals receiving D-ascorbic acid had hemorrhages about the joints to about the same extent as observed in Bioassay I.

Since changes in dental structures are a sensitive index of Vit. C deficiency(5), histochemical tests were made of the teeth of guinea pigs in Bioassay I. Dentin obtained from normal guinea pigs§ is tubular and stains red with both the Rinehart (Plate 1A) and alloxan-Schiff methods (Plate 1B). Dentin produced in the negative control group was atubular osteodentin, characteristic of scurvy. This abnormal dentin stained brilliant blue with Rinehart method (Plate 1C), indicating strong reactivity for acid mucopolysaccharides, and remained unstained or lightly stained with alloxan-Schiff method indicating lack of reactive proteins (Plate 1D). The small amount of bone produced during the scorbutic period was also strongly reactive with Rinehart stain for acid mucopolysaccharides and unreactive with alloxan-Schiff method for proteins. Thus the Rinehart stain proved to be a valuable method to detect the scorbutic state of guinea pigs.

Dentin produced in animals treated with L-ascorbic acid was tubular and stained the normal red with Rinehart (Plate 1E) and alloxan-Schiff methods (Plate 1F). Dentin produced in animals receiving D-ascorbic acid was also normal in that it had tubular structure and stained red with Rinehart (Plate 1G) and alloxan-Schiff methods (Plate 1H). However, predentin in this group usually reacted abnormally since it still retained the blue stain with Rinehart method (Plate 1G), and was unreactive with alloxan-Schiff method (Plate 1H).

Conclusion and summary. 1) Some activities of L-ascorbic acid can be replaced by D-ascorbic acid when administered in relatively small doses under conditions of these experiments. For instance, weight and survival of scorbutic guinea pigs can be maintained with D-ascorbic acid, but hemorrhages about the joints are not prevented. The most striking effect of D-ascorbic acid was revealed with histochemical studies of teeth. The dentin produced in animals receiving D-ascorbic acid was normal in morphology and staining properties. However, the Vit. C effect of D-ascorbic acid was not complete in that predentin formed in these animals was still abnormal in its reactivity to acid mucopolysaccharide stain though it was eventually converted to normal staining dentin. 2) These results furnish further evidence for multiple actions of Vit. C; those that are specific, for which only L-ascorbic acid will serve, and those that are non-specific in which D-ascorbic acid and presumably other compounds with the same

§ Guinea pigs which had been maintained on lettuce and Rockland Farm guinea pig diet, *ad lib.*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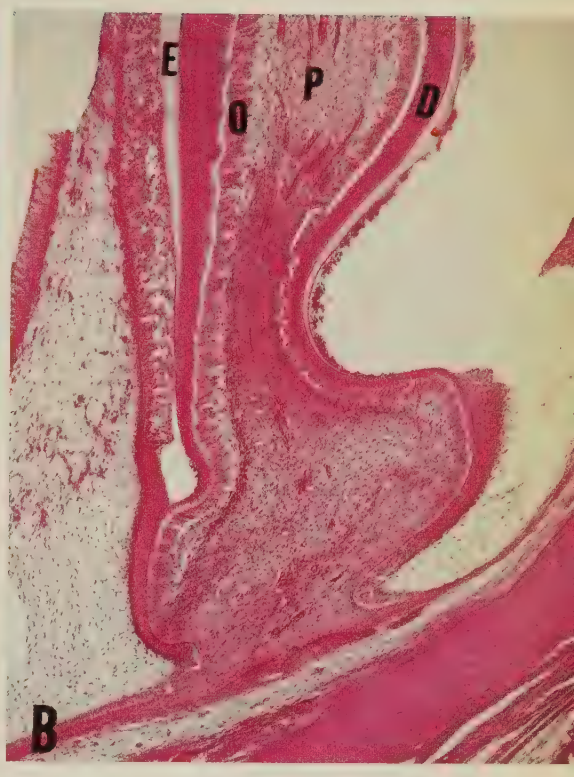
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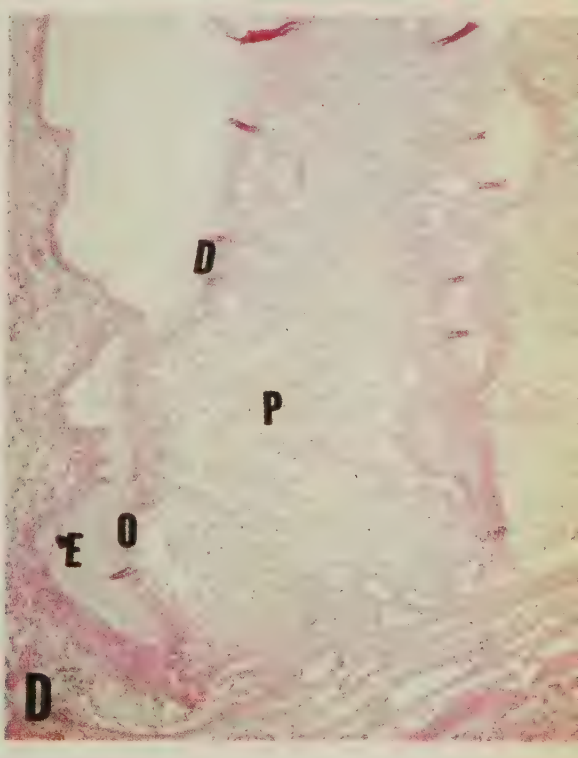
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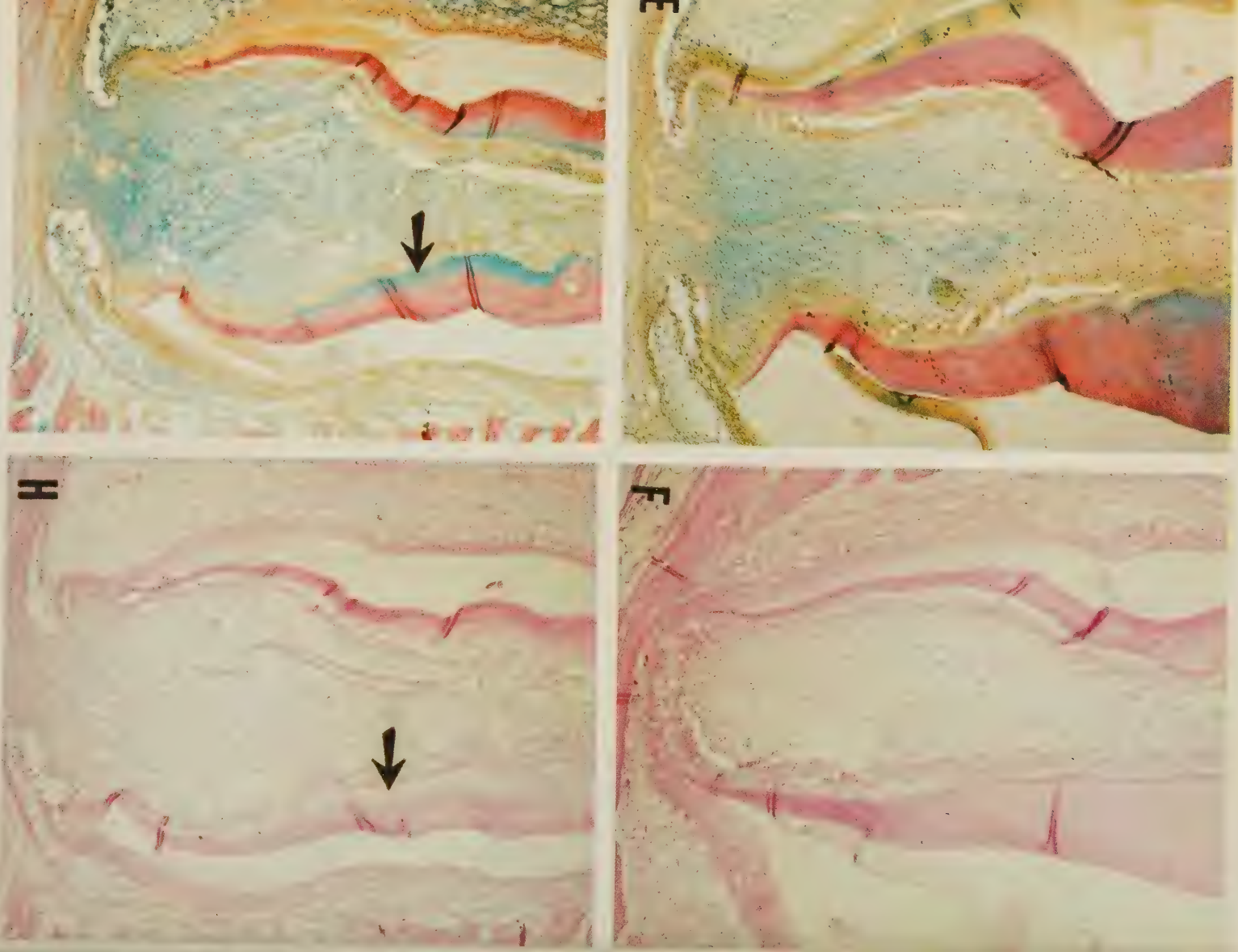
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E. Apical portion of a molar root from a guinea pig treated with L-ascorbic acid. Rinehart stain. Note normal morphology and staining characteristics of dentin formed during restoration period. × 73.

F. Apical portion of a molar root from a guinea pig treated with L-ascorbic acid. Alloxan-Schiff stain. Note normal morphology and staining characteristics of dentin formed during restoration period. × 73.

G. Apical portion of a molar tooth from a guinea pig treated with D-ascorbic acid. Rinehart stain. Note normal morphology of dentin and predentin. Note also that predentin (arrow) tends to retain the stain for acid mucopolysaccharides, whereas predentin in Plate IE does not. Dentin stains normally. × 73.

H. Apical portion of a molar tooth from a guinea pig treated with D-ascorbic acid. Alloxan-Schiff stain. Note normal morphology of dentin and predentin. Note also that predentin (arrow) tends to remain unstained, indicating lack of reactive proteins, while dentin stains normally. × 73.

redox properties can substitute. Separation of biological activities of Vit. C has been indicated by studies with D-araboascorbic acid in guinea pigs(4) and with D-ascorbic acid in monkeys(11). One of the best examples of non-specific role for the vitamin has come from studies of tyrosine metabolism in which L-ascorbic acid can be replaced by various other structurally unrelated compounds that are susceptible to oxidation and reduction(12, 13). Further studies with D-ascorbic acid in guinea pigs may throw light on the nature of non-specific functions of the vitamin.

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Use of Bromelin to Demonstrate Erythrocyte Antibodies.* (24828)

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Technics to demonstrate circulating incomplete erythrocyte antibodies fall into 3 categories: (A) coating cells and suspending them in media containing anisometric molecules (albumin, etc.)(1), (B) an indirect Coombs' test(2), (C) pretreating erythrocytes with proteolytic enzymes (trypsin, papain, ficin)(3). The first technic is generally inadequate in range of activity and sensitivity. The Coombs' procedure is sensitive and possesses widest range of activity. However, a prozone effect, high expense, and the tedious nature of the test make it undesirable. Pretreating with proteolytic enzymes eliminated the prozone effect, but the tedious procedure persisted. By prior activation of papain with L-

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cysteine, a technic was derived which permits direct mixing of enzyme, antibody, and target erythrocyte(4). Several problems are encountered with this method. The necessity of incubating L-cysteine with papain and storing aliquots frozen is clumsy. Marked digestion and color changes of erythrocytes may make end points difficult to read. A prolonged incubation time and sensitivity of the procedure have contributed little beyond that obtained with Coombs' technic. Major flaws in this technic are inability to demonstrate certain antibodies (MNS, Duffy, Kidd, Sutter)(5) and the lack of effect in demonstrating the incomplete antibody fixed to the erythrocyte. A new reagent and technic is now reported which possesses certain advantages over those previously used. Studies were made of a group of proteolytic enzymes derived from pineapple and supplied in a concentrated powdered form as bromelin.† Untreated isotonic solutions of this material could be mixed directly with sera containing incomplete antibodies and erythrocytes, leading to rapid, sharp, and sensitive agglutination reactions.

Methods. Various human sera containing incomplete anti-D (Rho) antibodies were serially diluted with isotonic saline. One drop of a 4% saline suspension of unwashed D (Rho) positive erythrocytes was mixed with 0.1 ml of diluted sera. One drop of bromelin solution was added directly to this mixture and macroscopic agglutination was determined after centrifugation for one minute at 1000 rpm. Optimum reaction time for this bromelin test was determined by incubating these mixtures at 37°C for varying periods prior to centrifuging. Sensitivity was determined immediately upon mixing at room temperature and after incubating for 5, 10, 15, 30, 45, and 60 minutes. An optimum bromelin reagent was determined as follows. Bromelin was dissolved in 9 parts saline and 1 part phosphate solution buffered at pH 8.6, 7.0, 6.2, 5.5, and 4.5. Concentration of the bromelin solutions was varied at 0.05%, 0.1%, 0.5% and 1%. The effect of preincubation in activating the bromelin solutions was determined by incubating solutions for one hour at 37° prior to testing. A standard bromelin test was therefore devised using unincubated reagent containing 0.5% bromelin buffered at pH 5.5. A test solution using one drop of bromelin, 0.1 ml of antibody-containing sera, and one drop of 4% saline suspension of unwashed erythrocytes was employed. Erythrocytes were chosen with an antigenic structure against which the antibody was directed. Erythrocytes not containing this antigenic group were used as controls. This mixture was immediately centrifuged one minute at 1000 rpm and inspected for macroscopic agglutination. A similar tube was incubated 15 minutes at 37°C for warm antibodies and at 4°C for cold antibodies, centrifuged and read in the same fashion. Stability of the bromelin reagent in this test procedure was determined after storage at 4°C, after storage at 4°C with 0.1% sodium azide, and after storage frozen at −20°C. Sensitivity and range of activity of these 2 bromelin procedures were compared to standard indirect Coombs' test and the activated papain-cysteine procedure as described by Stern(6). All warm-acting antibodies were incubated at 37°C and cold acting antibodies at 4°C, for one hour in Combs' and saline procedures, and for 30 minutes in the activated papain-cysteine procedure. Various human sera were tested as controls and 72 sera containing identified incomplete antibodies and 54 sera containing identified saline antibodies were investigated. Erythrocytes from 11 cases of auto-immune hemolytic anemia were studied. Each case was tested in direct Coombs' procedure and compared to various bromelin tests. A standard test was used consisting of one drop of bromelin added to 2 drops of 2% suspension of test erythrocytes. One tube was immediately centrifuged for one minute at 1000 rpm and read macroscopically for agglutination. A similar tube was incubated 15 minutes at 37°C before being centrifuged and read. The 2 test procedures were done using erythrocytes suspended in their own serum, saline suspensions of washed erythrocytes, and suspensions of washed erythrocytes resuspended in fresh, normal, ACD-collected AB plasma.

Results. Optimum reaction time for the bromelin test was 15 minutes incubation. The

† Supplied by Takamine Lab., Clifton, N. J.

immediate test, done without incubation, lowered sensitivity approximately one tube in titer studies. Similarly, increasing incubation time tended to decrease sensitivity of the reaction. Lowering the pH of the bromelin solution resulted in progressive increase of sensitivity, with optimum values obtained at pH 5.5. Increasing concentrations of bromelin progressively increased sensitivity of the reaction. Maximum values were obtained with 0.5% solutions; 1% solutions had a similar range of sensitivity. Preincubation of various bromelin solutions offered no increased titer advantages. The standard reagent used throughout remainder of this study was a 0.5% solution buffered to pH of 5.5. This solution maintained its potency approximately one month when stored at 4°C. By adding 0.1% sodium azide to the reagent, potency was maintained for over 2 months, when stored at 4°C.

Table I summarizes representative titer studies of incomplete and saline antibodies tested in the 4 standard procedures. In general, the following was noted: The indirect Coombs' procedure was frequently the least sensitive, with the papain-cysteine test usually increasing the titer by one tube. The 15-minute bromelin test often increased titers by 2 to 4 tubes over the indirect Coombs' test. The immediate bromelin test lowered titers, but was usually more sensitive than the indirect Coombs' and papain-cysteine procedures. Of particular interest is the sensitivity of the immediate bromelin test, performed at room temperature, in demonstrating cold-acting antibodies.

TABLE I. Sensitivity of Bromelin Immediate, Bromelin 15 Min., Papain-Cysteine, and Indirect Coombs' Tests with Incomplete and Saline Antibodies.

Antibody	Test titers: Bromelin, immed.	Bromelin, 15 min.	Papain-cysteine	Coombs'
Incomplete antibody				
Anti-D	1:512	1:1024	1:256	1:128
"	1:64	1:64	1:8	"
-E	1:32	1:128	1:16	1:8
-c	1:64	"	1:32	1:16
-K	"	"	1:16	1:8
-S	1:16	1:64	0	"
Saline antibody				Saline
Anti-A*	1:16	1:128	1:16	1:16
-B*	1:32	1:256	"	1:8
-D	1:256	1:512	1:256	1:64
-C	1:1024	1:2048	1:64	"
-P*	1:8	1:32	1:16	1:4
-Le^a*	1:4	1:8	1:2	1:2
-Tj^a*	1:32	1:128	1:32	1:32

* Cold acting antibodies. All tests performed at 4°C except the immediate bromelin procedure.

TABLE II. Positive Reactions of Incomplete and Saline Antibodies in An Immediate and 15-Min. Bromelin Test, a Papain-Cysteine Procedure, and the Indirect Coombs' Test.

Antibody	No.	Tests: Bromelin—Immed.	Bromelin—15 min.	Papain-cysteine	Coombs'
Normal sera	600	0	0		
Incomplete antibodies					
Anti-D	35	35	35	35	35
-C	1	1	1	1	1
-E	2	2	2	2	2
-c	4	4	4	4	4
-e	2	2	2	2	2
-V	1	1	1	0	1
-S	2	2	2	0	2
-U	1	1	1	0	1
-K	8	8	8	8	8
-k	2	2	2	2	2
-Fy^a	9	7*	7*	0	9
-Jk^a	3	3	3	0	3
-Vel	1	1	1	1	1
-Js^a	"	0*	0*	0	"
Saline antibodies					Saline
Anti-A†	17	17	17	17	17
-B†	"	"	"	"	"
-D	8	8	8	8	8
-C	2	2	2	2	2
-E	1	1	1	1	1
-Le^a†	4	4	4	4	4
-P†	"	"	"	"	"
-Tj^a†	1	1	1	1	1

* Negative reactions were converted into positive reactions by using plasma suspended erythrocytes.
† All tests except the bromelin-immed. were performed after incubation at 4°C.

Table II summarizes results of testing normal sera and sera containing identified incomplete or saline antibodies by the 4 standard procedures. Six hundred normal sera were tested in the 2 bromelin procedures without a false positive reaction. In addition, no false reactions were encountered with the negative erythrocyte controls, indicating specificity of the reaction. Seventeen sera contained an antibody identified by the Coombs' procedure and not demonstrated by the papain-cysteine procedure. With the 2 bromelin tests, identi-

TABLE III. Agglutination Reactions of Auto-Antibody Coated Erythrocytes in Acquired Hemolytic Anemia; Effect of Washing the Cells and Resuspension in Normal Plasma.

	Tests			
		Bromelin, 15 min.		
Disease	Coombs'	Unwashed	Washed	Re-suspended
SLE	2+	2+	—	1+
IM	1+	1+	—	"
IHA	"	"	—	"
"	2+	3+	—	2+
"	3+	"	—	"
"	2+	4+	—	"
CLLL	3+	3+	—	"
"	2+	"	—	"
"	3+	"	—	"
"	2+	"	—	"
"	"	4+	—	"

SLE = Systemic lupus erythematosus. IM = Infectious mononucleosis. IHA = Idiopathic hemolytic anemia. CLLL = Chronic leukemic lymphocytic leukemia.

cal results were obtained; 14 of these antibodies were detected. Two of 9 anti-Fy^a and the single anti-Js^a were not detected in the standard procedure. However, when erythrocytes were suspended in fresh, normal ACD-collected AB plasma, positive reactions were obtained.

Table III summarizes use of a 15-minute bromelin test in demonstrating presence of auto-antibodies coating erythrocytes. Essentially similar results were obtained with an immediate bromelin test. When erythrocytes were unwashed and suspended in their own sera, positive results were obtained. Washing these cells and suspending in saline eliminated the bromelin reaction. Resuspending washed, coated cells in normal, fresh, ACD-collected AB plasma returned the positive reaction.

Discussion. The above studies indicate that the described bromelin test has an apparent universal application, appearing effective with incomplete, saline, warm-acting, cold-acting, natural, and immune erythrocyte antibodies. In addition, the same reagent may be used to demonstrate coating of erythrocytes by antibodies. The reagent used is stable at 4°C for at least 2 months when 0.1% sodium azide is added, and cost of the material is negligible for a standard test. The simplicity of the test makes errors in procedure very unlikely. In addition, agglutination reactions are sharp with precise macroscopic end points in titrations. The reaction is an immediate one without preincubation, washings, or incubation necessary. Sensitivity of the reaction is surprising, usually surpassing the indirect Coombs' procedure. Ability of the immediate test to detect both cold- and warm-acting antibodies eliminates the necessity of duplicate studies done at varied temperatures. The importance of such a test system in establishing a routine cross match cannot be overestimated.

An interesting phase of this reaction is still to be investigated. Three antibody-containing sera were not positive with the standard test. The 2 anti-Fy^a and the anti-Js^a which were not detected could be demonstrated when test cells were suspended in fresh ACD-collected plasma. A similar dependency on fresh plasma in this reaction was seen in demonstrating auto-antibodies coating erythrocytes. The nature of this plasma dependency is still under study.

Summary. Isotonic solutions of bromelin buffered to pH 5.5 were utilized in an immediate and 15-minute procedure to demonstrate antibodies directed against the ABO, Rh-hr, P, MNS, Kell, Duffy, Kidd, Lewis, Vel, and Sutter blood groups. In addition, a bromelin test detected erythrocytes coated with incomplete antibodies. This reaction appears to be dependent on presence of fresh plasma.

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Fate of Radiosulfate in Multiple Myeloma.* (24829)

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Evidence establishing presence of an abnormal protein-bound polysaccharide as one of the metabolic abnormalities associated with multiple myeloma has been reported from this and confirmed by other laboratories(1,2,3,4). Studies with radiosulfate in myeloma were initiated as part of a program designed to investigate mucopolysaccharide metabolism in this disease. This report describes the turnover, dilution, and excretion of inorganic radiosulfate in patients with myeloma and, for comparison, in patients with other malignant disorders.

Methods. Carrier-free S^{35} as sodium sulfate in doses ranging 0.867 to 2.07 millicuries was administered intravenously to 9 patients with multiple myeloma and 10 subjects with other neoplastic diseases (Table I). Assays of radioactivity were performed with a gas flow counter, since S^{35} is a pure, weak beta emitter (0.168 mev maximum energy; 87 days half-life). Suitable corrections for self-absorption and backscattering were made for specimen geometry adopted as well as for physical decay. The equipment was calibrated against S^{35} standard solutions supplied by the National Bureau of Standards. Venipunctures were performed at intervals of 5 minutes, one hr, 4 hr, 8 hr, 24 hr, and 48 hr following administration of radiosulfate. Blood specimens were placed in test tubes containing 2 drops of versene to prevent coagulation. Total urinary output was collected during first and second day following injection of S^{35}. *Calculations.* Concentration of S^{35} (% dose/liter) in whole blood and in plasma was determined (Fig. 1), as was the fraction of S^{35} dose excreted (% dose excreted) in urine during the first 2 days following S^{35} administration (Table I). The apparent space of dilution was calculated from blood and plasma data. This computation involved finding net concentration at zero-time by graphical extrapolation, to obtain the so-called zero-time radiosulfate space(5). The body surface of each participant was determined from body weight and height, using the conventional DuBois relationship.

Results. 1. *S^{35} blood levels.* S^{35} concentration in blood as a function of time falls rapidly (Fig. 1). This is true for both whole blood and plasma, and for multiple myeloma patients as well as other subjects. The initial whole blood maxima, obtained 5 minutes following injection of radiosulfate, range from 8.04% dose/liter to 15.7% dose/liter for multiple myeloma patients and from 6.12% dose/liter to 13.1% dose/liter for control subjects. The corresponding plasma concentrations at 5 minutes range from 7.38% dose/liter to 14.4% dose/liter for multiple myeloma and from 6.35% dose/liter to 13.4% dose/liter for the others. By 8 hours, however, these S^{35} levels dropped to almost a third of their respective initial values.

For 16 of 19 patients, whole blood and plasma S^{35} concentration data, when graphed semi-logarithmically, could be resolved into 3 distinct exponential components. Fig. 2 illustrates such a plasma concentration S^{35} curve, together with its 3 exponential decay rates, obtained from a multiple myeloma patient. From analysis of such plots, obtained from both whole blood and plasma concentrations, it appears that an initial rapid turnover, corresponding to half-disappearance time (T_1) of about an hour, governs the fall of S^{35} for the first few hours. For the ensuing hours of first day, the decrease is described by half-time (T_2) of approximately 3 hr. A third half-time (T_3), about 17 hr, characterizes the second day following the dose. Mean values of these various half-disappearance times for myeloma and other patients as derived from blood and plasma data are listed in Table II. For relating these half-disappearance times to corresponding turnover constants (k), the

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TABLE I. S^{35} Urinary Excretion and Zero-Time Whole Blood Spaces of Dilution in Patients.

Sex	Age	Diagnosis	% urinary excretion /24 hr 1st	% urinary excretion /24 hr 2nd	Space (liter)	Space $\frac{\text{liter}}{\text{Body surface}}$ $\left[\frac{\text{liter}}{\text{meter}^2}\right]$	Space $\frac{1 \times 100}{\text{Wt}}$ $\left[\frac{1 \times 100}{\text{kg}}\right]$
			Group A				
♀	52	Multiple myeloma	26.8	1.01	7.12	6.98	12.0
♀	57	*Idem*	69.6		12.0	7.15	18.6
♂	39	"	33.6	18.6	12.0	7.15	20.3
♂	54	"	60.3	17.2	10.4	5.20	12.5
♀	73	"	79.0	5.19	6.08	3.80	9.57
♂	73	"	39.4	9.10	8.00	4.15	11.3
♂	61	"	42.7	20.0	11.7	6.83	18.3
♂	74	"	54.4	7.30	7.50	4.84	15.5
♂	65	"	57.0	20.2	8.75	4.97	13.2
		Avg	51.4 ± 16.1*	12.3 ± 7.1	9.29 ± 2.16	5.67 ± 1.27	14.6 ± 3.5
			Group B				
♂	57	Hypernephroma	72.2	16.5	8.40	5.12	14.7
♀	33	Ca breast	39.8	12.1	13.7	7.34	15.9
♂	69	Ca sigmoid	51.8	11.6	12.2	6.17	15.1
♂	78	Osteogenic sarc.	71.2	9.91	9.85	6.20	16.6
♂	76	Ca sigmoid	80.2	9.10	9.20	5.44	15.5
♀	53	Ca breast	15.4	5.84	16.0	8.80	22.0
♀	41	Hodgkin's sarc.	46.7	6.50	15.2	9.50	27.6
♀	62	Ca breast	80.7	6.60	11.0	7.05	17.8
♀	46	Ca breast	62.1	12.1	7.40	5.52	18.7
♀	62	Met. bone dis., primary unknown		18.9	10.7	6.04	14.6
		Avg	57.8 ± 20.3	10.9 ± 4.08	11.4 ± 2.87	6.72 ± 1.39	17.9 ± 3.9

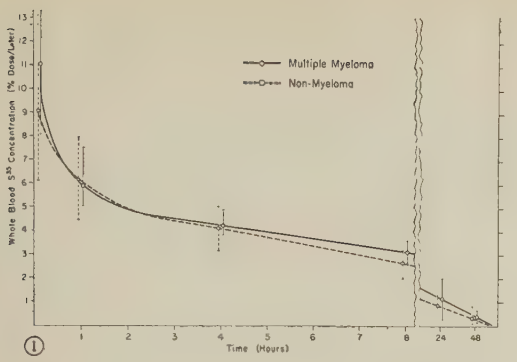
* Stand. dev.

approximate relationship $k = 0.693/T$ holds. For the 3 other patients (2 of whom had myeloma), the initial rapid rate (T_1) was absent; T_2 and T_3 were both present, in good agreement with values tabulated.

2. *S^{35} urinary excretion.* The combined first and second 24 hr urinary excretion of S^{35} range from almost 30% of dose to maximum of 90%, with no apparent difference between the 2 groups studied (Table I).

3. *Radiosulfate space of dilution.* The zero-time radiosulfate spaces and the ratio of these for whole blood and plasma spaces to body surface and to weight, were similar for myeloma patients and other subjects (Table I). Since concentration of S^{35} in whole blood does not differ appreciably from that in plasma, the respective spaces are almost identical, and, for brevity, only the whole blood radiosulfate spaces are tabulated.

Discussion. The turnover, spaces of dilution, and urinary excretion of S^{35}-sulfate in patients with multiple myeloma and other malignant disorders were similar. Although utilization of inorganic sulfate by the mammal is not fully understood, it is known that elementary sulfur cannot replace cystine or methionine for incorporation into tissue protein of the rat(6,7). Other studies, however, demonstrated that S^{35}-sulfate is utilized by the cytoplasm of the myeloid series of bone marrow cells(8,18). Also, there is a significant incorporation of S^{35}-sulfate in the sulfomucopolysaccharide of mammalian tissues such as cartilage, aorta, sclera, cornea, and intestinal mucosa(9,10,11,12). Indeed, Boström suggests that it may be possible to consider S^{35} sulfate metabolism as reflecting the metabolism of the whole molecule of sulfomucopolysaccharide(13). He demonstrated that for the rat, the principal uptake of S^{35} sulfate was by mucopolysaccharides containing ester sulfates, with a low, but significant uptake in taurine isolated from liver. There was no uptake in cystine or methionine(14). In the rat, Smith and coworkers have shown that about 40% of administered S^{35} sulfate activity was bound to a serum constituent having



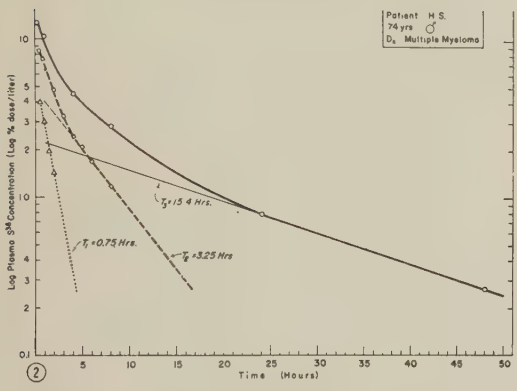


FIG. 1. Variation of whole blood S^{35} concentration with time. Individual points represent mean values in 9 patients with multiple myeloma and 10 patients with other neoplasms. Range (max and min values) is indicated by vertical lines extending from points.

FIG. 2. Resolution of a plasma S^{35} concentration curve into 3 exponential components. T_1, T_2, and T_3 are corresponding half-disappearance times.

the electrophoretic mobility of α_1-globulin; they suggest that the binding occurs in a protein bound sulfomucopolysaccharide(15).

The S^{35} concentration in blood and plasma, turnover times and fraction excreted are in general agreement with the findings of others for animals and normal man. The almost identical whole blood and plasma concentrations suggest that an appreciable amount of S^{35} enters the red cell, a conclusion in accord with data reported for the rat(16,17). Values of the various half-disappearance times (Table II) indicate a rapid turnover of administered radiosulfate. Our values for the intermediate half-time (T_2), and for radiosulfate space for both groups of patients are in close agreement with those reported for normal human subjects(5,19).

TABLE II. Initial, Intermediate, and Late Half-Disappearance Times Obtained from S^{35} Whole Blood and Plasma Concentration Curves.

	Avg half-disappearance times (hr)		
	T_1	T_2	T_3
Whole blood			
Multiple myeloma	.635 ±.205*	2.90 ±.274	15.9 ± 2.2
Other neoplasms	.778 ±.308	3.23 ±.44	17.4 ± 3.9
Plasma			
Multiple myeloma	1.05 ±.29	3.73 ±.36	17.0 ± 3.0
Other neoplasms	.981 ±.451	3.54 ±.64	17.9 ± 4.4

* Stand. dev.

It would appear that administered inorganic sulfate is rapidly turned over and quickly excreted with little, if any, being retained for tissue incorporation. The rapidly excreted S^{35} may be the original sodium sulfate or in the form of sulfomucopolysaccharide, which also is rapidly excreted after administration (personal communication, R. Soberman). In the human, one may conclude that only a small portion of the original dose is retained for utilization and for incorporation into sulfomucopolysaccharide, and, with the technics used, an increased retention could not be demonstrated in multiple myeloma as compared to other neoplasms.

Summary. Radiosulfur (S^{35}) as sodium sulfate was administered intravenously in doses ranging from 0.867 to 2.07 mc, to 9 patients with multiple myeloma and to 10 patients with other neoplasms. The fate of administered sulfate was apparently the same for both groups of patients. S^{35} was rapidly turned over and excreted; 30 to 90% appeared in urine within 48 hours. Blood levels of S^{35} closely paralleled those of plasma and decreased rapidly. It was possible to demonstrate that 3 exponential rates governed the disappearance of S^{35} from whole blood and plasma. The mean zero-time radiosulfate space of dilution was 9.29 ± 2.16 liters for patients with multiple myeloma and 11.4 ± 2.87 liters for those with other neoplasms. By technics employed, a preferential retention of sulfate could not be demonstrated in multiple myeloma as compared to other neoplasms.

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Effect of Close Arterial Injections of KCl on Gastric Potential Difference.* (25830)

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Numerous studies on a variety of organisms have shown that there is in general an inverse relationship between K^+ ion concentration of ambient fluid and magnitude of transmembrane potential difference (P.D.). In the frog skin(1), resting nerve and other tissues, it has been suggested that the potential difference is due primarily to K^+ ion gradient from inside of cell to the nutrient medium. There are exceptions to this general rule, for example, the findings of Tasaki *et al.*(2) with respect to K^+ ion gradient in fluids of inner ear, where the gradient is opposite to that predicted by the classical scheme. In the gastric mucosa, it has been suggested that the P.D. arises from EMF's associated with active transport of Cl^- ion(3,4). However, the possibility exists that the actual EMF of gastric mucosa is dependent on K^+ ion gradient from the cell to the interstitial fluid (ISF). Our primary purpose was to determine the effect of increasing K^+ ion concentration in ISF on gastric P.D. Because of systemic toxic effects of elevated plasma K^+ ion concentration, the method of close arterial injection was used. A preliminary report has been published(5).

Methods. Amytalized dogs were used with chambered gastric segment technic(6). Approximately 20 cm^2 of the secretory portion of stomach with blood supply intact, is placed in a lucite chamber. Two pairs of reversible electrodes were used, one pair for measuring P.D. and the other pair for sending current (1 ma/20 cm^2). P.D. was measured with Brown recording potentiometer. Direct current resistance was determined as change in P.D./unit of applied current(7). One branch of splenic artery was canulated for close arterial injections. All other branches except those going to stomach were ligated. Injections were made with motor driven syringe. All experiments were performed on resting stomachs with 0.16 M NaCl solution bathing the mucosal surface.

Results. Fig. 1 illustrates a typical experiment showing effect of injection of several iso-

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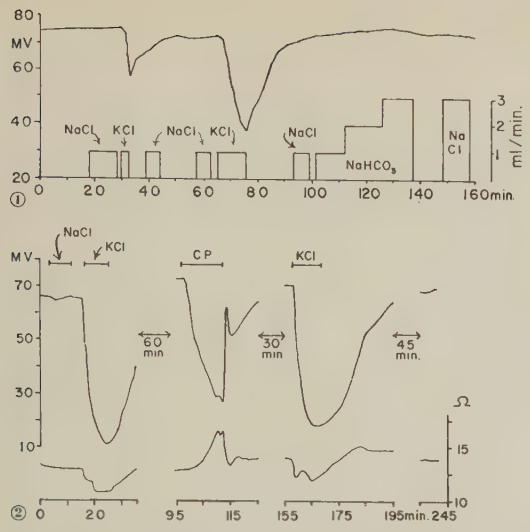

FIG. 1. Potential difference across the mucosa (serosa positive in an external circuit) as a function of time is shown by solid curve. Block denotes close intra-arterial inj. of isotonic electrolytes with the height denoting rate.

FIG. 2. Upper curves show potential difference as a function of time and the lower the direct current resistance in ohms for 20 cm^2. Lines at the top indicate periods of close arterial inj. (1 ml/min. for each experiment) or occlusion of blood supply (CP).

tonic (0.16 M) solutions on P.D. The right hand ordinate gives rate of close arterial injection in ml/min. NaCl and $NaHCO_3$ injections did not result in significant change in P.D. but KCl injections resulted in marked decrease in P.D.

Injection of KCl resulted in very marked blanching of mucosa not observed with other electrolytes even at rates of 5 ml/min. Blanching occurred when the P.D. started to fall. Following termination of KCl injection, the normal pink color of the mucosa usually returned immediately and P.D. started rising. In some experiments, the stomach remained blanched for varying periods following cessation of KCl injection and P.D. did not start increasing until pink color returned. On the assumption that blanching indicates drastic decrease in blood flow through the mucosa, the possibility was considered that decrease in P.D. was due to anoxia since it has been shown that interruption of blood supply results in decrease in P.D.(8). Following interruption of blood supply, the direct current resistance of the stomach increases. Obviously if the effect of KCl injections is due solely to lack of blood flow, then resistance should increase. Fig. 2 shows that the effect of KCl injections was a decrease in resistance. The magnitude of decrease in resistance was a function of the magnitude of decrease in P.D. For those experiments in which injection was continued until the P.D. fell below 20 mv. average decrease in resistance was 2 ohms for 20 cm^2 (8 experiments range 1.2 to 3.2). During recovery, P.D. and resistance increase and return to approximately their original levels.

The effect of clamping the pedicle is illustrated in the middle experiment Fig. 2. Duration of clamping is indicated by the line labelled CP. Following the experiment on interrupting the blood flow, the stomach still behaves in typical fashion with KCl injections as seen in last experiment in this Figure.

The effect of KCl injection differs from that of clamping the pedicle. Apart from the difference in direction of the resistance change, P.D. decreases more rapidly following KCl than with clamping of pedicle. The rate at which both resistance and P.D. return to normal is greater following release of clamped pedicle than following cessation of KCl injection. In spite of these differences, it is not possible to conclude that the effect of KCl injection is not due in part to anoxia. In some experiments, when blanching persisted longer than shown in the Figures, resistance started rising. In one experiment, blanching persisted for 19 minutes after cessation of KCl injection and P.D. fell to approximately zero. After a typical fall in resistance (2 ohms), the resistance 14 min from start of injection started to increase and increased by 15 ohms in the following 19 minutes. At this time, close arterial injection of 0.16 M NaCl resulted in reestablishment of normal color of the mucosa and a response of P.D. and resistance essentially the same as that seen after reestablishing the blood supply following a period of interrupted blood flow, *i.e.*, similar to results shown in middle experiment of Fig. 2.

It is apparent that a quantitative study of the effect of changes in K^+ ion concentration in ISF, in preparations with intact blood supply is beset with difficulties. It was planned

originally to estimate K^+ ion concentration in mucosal ISF by determination of K^+ ion concentration in venous outflow from the segment, after steady state conditions were established. Blood flow through the segment in absence of intra-arterial injection ranges from 7 to 12 ml/min (collection of entire outflow after heparinization). However, because of blanching, it is obvious that K^+ ion concentration in plasma of the venous outflow would not be a reliable index of K^+ ion concentration in ISF of the mucosa. Smaller concentrations of KCl (plus NaCl for isotonicity) and lower injection rates were tried but there was no fall in P.D. until the stomach blanched. With lower concentrations and longer periods of injection, there was a tendency for resistance to increase after the initial decrease (probably the result of prolonged anoxia of mucosa).

An attempt was made to prevent blanching by injection of a vasodilator (histamine, methacholine chloride or sodium nitrite) along with KCl. These agents even in high concentration (when injected close arterially with KCl, resulted in lowering of mean carotid blood pressure) did not prevent blanching.

Discussion. A marked vasoconstrictor action of elevated plasma K^+ ion concentration has been previously reported(9). Although it is not possible to arrive at a clear cut conclusion on the relationship between P.D. and K^+ ion concentration in ISF, the results are compatible with the idea that increase in K^+ ion concentration in ISF results in decrease in both P.D. and resistance. Mond(10) had previously reported that in the perfused frog stomach, an elevation of K^+ ion results in decrease in P.D. However, our results force one to consider the possibility that in Mond's experiment, the high K^+ ion may have diverted the perfusion fluid from the mucosa with a consequent decrease in O_2 supply and that part of the observed effect may have been due to anoxia. Harris and Edelman(11) on the other hand found that P.D. of the secreting *in vitro* frog's stomach is inversely related to K^+ ion concentration over range of 1 to 9 meq of K^+ ion/liter. One might be tempted to assume that K^+ ion gradient from cells to ISF is solely responsible for the P.D. However, other evidence indicates that there may be another EMF dependent on presence of Cl^- ion.

Summary. We determined the effect of increasing K^+ ion concentration of interstitial fluid on the potential difference across the resting dog's stomach. The chambered gastric segment preparation was used and because of toxic systemic effects of elevated plasma K^+ ion concentration, local plasma K^+ ion concentration was elevated by close arterial injection. Close arterial injection of KCl resulted in a decrease in P.D., a decrease in resistance, and a marked blanching of the mucosa. Control experiments with NaCl and $NaHCO_3$ did not result in blanching and had relatively little influence on P.D. or resistance. That the effects of close arterial KCl injections are not the result of anoxia alone is suggested by the occlusion of blood supply producing a decrease in P.D. but an increase in resistance.

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Effects of Freeze-Thawing and Storage on Ultracentrifugal Properties of Human Serum Lipoproteins. (24831)

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Investigations on the effects of freezing on human serum lipoproteins have appeared in the literature. In one report a comparison of the extractability of lipids with ether was made between liquid and frozen lipoprotein solutions(1). In other studies, extent of lipoprotein change due to freezing and storage was investigated by noting alteration in solubility(2,3). The effects of freeze-thawing and storage on ultracentrifugal properties of lipoproteins have not been studied except for observations relative to one lipoprotein group (4). These effects represent a problem that must be solved before determination of the clinical importance of serum lipoproteins, whenever those conditions are involved. This paper summarizes the results of our investigation into the problem.

Material. Human sera from male adults with elevated concentration of lipoproteins were used. Sera were distributed in samples by pipetting 2 ml of each serum into 10-ml screw-cap vials, which were carefully sealed under nitrogen. *Freezing processes.* For rapid freezing (r.f.), the vials were dipped into dry ice and acetone for 50 sec. For slow freezing (s.f.) vials were set in deep freeze 1 hr. In both processes, final temperature was $-28° \pm 1°C$ as checked with a copper-constantan thermocouple. *Thawing.* For rapid thawing (r.th.), vials were placed in water bath at $+ 37°C$ for 120 sec. For these conditions serum reached a temperature of $24° \pm 1°C$. For slow thawing (s.th.) vials were left at room temperature ($24 \pm 2°C$) for 1 hr, after which temperature equilibrium had usually been reached. *Storage.* Three ranges of storage temperature were studied: (a) between $-30°$ and $-26°C$; (b) between $-5°$ and $0°C$; and (c) between $0°$ and $+4°C$. At temperatures between $-30°$ and $-26°C$ sera appeared constantly and thoroughly frozen in contrast to temperatures between $-5°$ and $0°C$; in this range sera were not always thoroughly frozen. *Ultracentrifugation.* Isolation and analysis of serum lipoproteins were performed by previously described procedures(5). Isolation was carried out in Spinco Model L preparative ultracentrifuge. For isolation of low-density lipoproteins (LDL) serum was diluted with equal volume of NaCl solution ($\rho\ 20°/4° = 1.118$ g/ml). For isolation of total high-density lipoproteins (HDL), serum was diluted with equal volume of $NaNO_3$ in D_2O solution of $\rho\ 20°/4° = 1.390$ g/ml, yielding a background solution of $\rho\ 20°/4° = 1.20$ g/ml. Determinations of flotation rates and concentrations were carried out in Spinco Model-E analytical ultracentrifuge. Lipoproteins were classified as follows: LDL: $S^{\circ}_{f(1.06)}$ 100-400, 20-100, 12-20, 0-12 (flotation rates were corrected for concentration and Johnston-Ogston effects); HDL: lipoproteins with hydrated densities in range between 1.075 g/ml and 1.15 g/ml. Solution densities were determined with 1-ml pycnometer.

Standard errors of measurement are as follows: S°_f 0-12: 19; 12-20: 16; 20-100: 22; 100-400: 34; HDL: 10. These standard errors can be expressed as percentage errors by dividing them by their respective means: 7%, 2%, 10%, 12% and 4%.

Results. Table I presents average results of freeze-thawing of 2 sets of sera. Repeated successive cycles (from one to 3 times) of either rapid or slow freeze-thawing of serum produce no significant changes in either concentration or flotation rate of the major classes of lipoproteins.

Once it was established that changes resulting from a single freeze-thawing process were minimal, the effects of storage in deep-frozen

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TABLE I. Freeze-Thawing of Serum. Serum concentration in mg %.

	Avg for 4 sera		Avg for 3 sera	
	Orig.	r.f. r.th. (3×)	Orig.	s.f. s.th. (3×)
$S^{\circ}_{f(1.06)}$ 100-400	239	237	282	281
20-400	216	213	240	235
12- 20	55	55	56	59
0- 12	317	315	325	319
Total LDL	827	820	904	893
HDL	280	291	292	282
Total lipop't'n	1107	1111	1196	1175

state were studied in combination with rapid and with slow freezing and thawing processes. Table II presents average of results for 2 sera for each condition of storage. The discussion of this Table is in line with that of Table III. Table III presents average results of comparative study of storage of 3 sera at temperatures between –30° and –26°C, between –5° and 0°C, and between 0° and +4°C. (Results of analyses intermediate to periods shown in Tables are omitted for brevity.)

At temperatures between –30° and –26°C, lipoproteins with flotation rates above S°_{f} 20 were more rapidly degraded and showed more extensive degradation over comparable periods of storage than lipoproteins with flotation rates less than $S^{\circ}_{f(1.06)}$ 20. In contrast, $S^{\circ}_{f(1.06)}$ 0-20 as well as HDL lipoprotein classes showed relative stability to storage between these temperatures. After 6 months, the maximum change observed in the HDL class was 30% decrease in concentration.

At temperatures between –5° and 0°C all lipoprotein classes appeared to be stable for at least 28 days. Thereafter, S°_{f} 0-20 and HDL lipoprotein classes appeared to be more resistant to degradation than the other lipoprotein classes. After 21 to 28 days of storage, the HDL lipoprotein class showed some qualitative changes not evident in Table III. These changes were a broadening of lipoprotein distribution accompanied by increase in flotation rate of the major peak.

At temperatures between 0° and +4°C, all lipoprotein classes appear to be stable for at least 14 days. Thereafter, there is no consistent pattern of degradation. Qualitative changes frequently appear as concentration increases and decreases within any broad lipoprotein class, without any net concentration change of that class. After 7 to 14 days of storage, the HDL lipoprotein class showed a broadening of lipoprotein distribution and increase in flotation rate of the major peak.

Conclusions. 1) Storage of serum at a temperature between –5° and 0°C yielded stable values for human serum lipoproteins for at least 4 weeks. 2) Storage of serum between 0° and +4°C for as long as 2 weeks also revealed no quantitative change in lipoprotein pattern. 3) Storage for even a few days at –28°C was associated with alterations in relative proportions of lipoprotein fractions. It is interesting to mention that under similar treatment, lipoproteins in salt solutions undergo faster degradation than those in serum.

Summary. The effects of freeze-thawing and of storage on ultracentrifugal characteristics of human serum lipoproteins have been studied. Two different rates of freezing and thawing have been used. Storage has been carried out at 3 different temperatures: be-

TABLE II. Storage of Serum at Temperature between –30° and –26°C. Serum concentration in mg %.

	Avg for 2 sera							
	r.f.—storage—r.th.				s.f.—storage—s.th.			
Days of storage→	Orig.	7	180	360	Orig.	7	180	360
$S^{\circ}_{f(1.06)}$ 100-400	274	212	162	51	137	140	72	Traces
20-100	230	152	129	45	195	160	68	Traces
12- 20	44	37	45	33	67	51	47	31
0- 12	220	198	217	220	314	274	252	224
Total LDL	768	599	553	349	713	625	439	235
HDL	258	255	264	203	247	270	203	189
Total lipoprotein	1026	854	817	552	960	895	642	444

TABLE III. Storage of Serum at Temperatures between –30° and –26°C, –5° and 0°C, and 0° and +4°C. Serum concentration in mg %.

		Avg for 3 sera								
		s.f.—storage—s.th.								
		–30° and –26°C			–5° and 0°C			0° and +4°C		
Days of storage→	Orig.	7	28	128	7	28	178	7	78	178
$S^{\circ}_{f(1.06)}$ 100-400	250	235	187	98	258	267	151	253	230	108
20-100	356	137	124	87	363	350	140	348	301	208
12- 20	41	24	25	24	39	41	32	42	44	40
0- 12	270	232	247	227	272	284	230	278	272	233
Total LDL	917	628	583	436	932	942	553	921	847	589
HDL	232	236	237	199	227	224	166	257	216	195
Total lipoprotein	1149	864	820	635	1159	1166	719	1178	1063	784

tween –30° and –26°C, between –5° and 0°C, and between 0° and +4°C. Adequate preservation of lipoproteins stored as serum at a temperature between –5° and 0°C was maintained for 4 weeks, between 0°C and + 4°C for 2 weeks, and between –30° and –26°C for a few days.

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Changes in Renal Tissue Composition Induced in Rabbits by Various Intravenous Doses of Mercaptomerin Sodium.* (24832)

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Previous studies(1,2) have shown that the organic mercurial diuretic agent, mercaptomerin sodium, localizes in kidneys when administered parenterally to animals or human subjects. The renal histopathologic effects of the organic mercurials are well known, but the nature of the changes in tissue composition is still obscure. The purpose of the present study in normal rabbits was to determine what changes in water, sodium and potassium contents of renal tissue were produced by intravenous injection of various doses of mercaptomerin sodium.

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Material and methods. Forty-four normal albino male rabbits were kept in individual metabolism cages and fed a stock diet of compressed pellets. Tap water was given without restriction. Six control animals were not injected. Six animals received intravenous injection of mercury of 0.5 mg/kg of body weight; 9 animals were given 1 mg/kg; 5 were given 5 mg/kg and 6 each were given 10, 15 and 20 mg/kg. The mercury was given as commercial lyophilized mercaptomerin sodium (Thiomerin), dissolved in 1 or 2 ml of water immediately prior to injection. All test animals were killed by air embolism 48 hours after administration of the mercurial. Both kidneys were removed and weighed, and the cortices were then separated from the medul-

TABLE I. Renal Water, Sodium and Potassium Content in Rabbits Given Various Intravenous Doses of Mercaptomerin Sodium.

I.V. dose of mercaptomerin (mg Hg/kg)	No. of rabbits in group		Cortex Na (meq/kg wet tiss.)	Cortex K (meq/kg wet tiss.)	Cortex H_2O (%)	Medulla Na (meq/kg wet tiss.)	Medulla K (meq/kg wet tiss.)	Medulla H_2O (%)
0	6	Mean	68.92	60.62	78.93	103.11*	57.29	81.46*
		S.D.	9.30	5.70	1.20	14.40	6.80	3.10
		No.†	20	20	20	16	16	16
.5	6	Mean	70.45	66.96*	77.20*	85.89*	66.04*	80.05
		S.D.	5.42	6.87	1.17	13.05	4.91	1.63
		No.†	22	22	22	22	22	22
1.0	9	Mean	65.16	62.29	79.28	99.48	60.71	81.81
		S.D.	7.62	3.48	1.89	25.34	5.78	2.55
		No.†	30	22	30	30	22	30
5.0	5	Mean	64.69	56.01	80.04*	83.20*	56.49	80.93
		S.D.	4.92	5.21	1.00	23.10	6.02	1.60
		No.†	16	16	16	16	16	16
10.0	6	Mean	77.45*	42.55*	82.60*	71.55*	48.12*	80.02
		S.D.	3.40	2.78	1.56	8.02	5.36	3.03
		No.†	22	22	22	20	20	20
15.0	6	Mean	76.58*	42.55*	82.65*	66.36*	47.33*	82.55
		S.D.	5.66	6.82	1.71	5.22	4.37	1.10
		No.†	22	22	22	19	19	19
20.0	6	Mean	76.29*	42.64*	83.35*	67.17*	50.78*	83.10
		S.D.	4.65	4.65	.90	4.40	4.70	.86
		No.†	20	20	20	20	20	20

* Significant difference when compared with respective mean control values. P = less than 0.01.

† No. of specimens analyzed.

las by gross dissection. In most instances, 2 samples from the medulla and 2 from the cortex of each kidney were placed in test tubes of known weight. The samples weighed approximately 1 g each. The test tubes were then dried at 110°C until constant weights were reached. After dry tissues had been pulverized with glass rod, 10 ml of demineralized distilled water was added to each tube. The tubes were shaken, stoppered, and stored in refrigerator 6 days, then centrifuged. Sodium and potassium contents of supernatant fluid were determined by lithium internal standard method of flame photometry. *Statistical analysis.* The significance of difference between mean values was tested by the formula for comparing 2 groups with a different number of observations in each group(3).

Results. (Table I). In 6 normal rabbits used as controls, the mean concentration of sodium in the medulla (103.1 meq/kg wet wt of tissue) was considerably higher than that found in the cortex (68.9 meq/kg). Water content of medulla (81.5%) was significantly higher than that of the cortex (78.9%).

Administration of mercury 0.5 mg/kg produced the following significant changes: increase in potassium content and decrease in water content in the cortex, decrease in sodium and water content and increase in potassium content in the medulla. Tissue changes at 1 and 5 mg/kg of mercury were not striking except for the decrease in medullary sodium content. Injection of mercury in doses of 10, 15 or 20 mg/kg resulted in a significant increase in cortical sodium concentration and water content and a decrease in cortical potassium concentration. Medullary concentrations of sodium and potassium were significantly and strikingly decreased following injection of mercury in doses of 10, 15 or 20 mg/kg. Water content of the medulla was not significantly altered by these doses of mercury.

Comments. Ullrich and Jarausch(4) have previously shown in dogs that the tissue sodium concentration in thirsted animals increases progressively from the cortex to the

outer zone of the medulla and to the tip of the papilla. The rather high concentration of sodium in the medulla of our control animals given water without restriction as compared with that in the cortex, with very little difference in potassium concentration or water content, may be attributed theoretically to one or more of the following factors: 1) a higher proportion of extracellular fluid in medulla than in cortex, 2) presence of tubular fluid and urine, or 3) a high intracellular concentration of sodium within medullary cells. Since concentration of sodium in serum of normal rabbits is 131.9 ± 7.2 meq/l(5), and since not more than 55% of the medulla is thought to be composed of interstitial tissue (6), it is difficult to account for high concentration of sodium in the medulla on the basis of extracellular fluid alone. It cannot be determined from the present study whether the high medullary concentration of sodium is due to tubular fluid or to intracellular accumulation. Ljungsberg(6) has previously concluded from a histotopochemical study in rats, that chloride is located within the cells of the collecting tubules. Although he attributed this concentration to active chloride reabsorption, Smith(7) has stated that active sodium reabsorption is more probable. Radioautographic studies with Na^{22} in rats(8) have shown that maximal concentration of radioactivity occurs in cells of the renal collecting duct. Data from our study in rabbits are compatible with the interpretation that tubular cells may concentrate sodium.

Diuretic doses of mercury (below 10 mg/kg) appear to have produced dehydration and concentration of potassium in both cortex and medulla, with a decrease in sodium concentration in the medullary tissue. Previous studies with Na^{22}(8) have shown that diuretic doses of an organic mercurial produce autographic changes in the cortex, attributed to alterations in the proximal convoluted tubules, and in the medulla, thought to be due to functional changes in cells of the collecting ducts.

A single intravenous dose of 10 mg of mercury or more/kg body weight is nephrotoxic. In our study on rabbits, doses of 10, 15 and 20 mg/kg produced in the cortical tissue electrolyte changes compatible with cellular damage —a decrease in potassium and an increase in sodium concentration and water content. In the medulla, these dosages caused a decrease in sodium and potassium contents, changes which might be due to a less severe degree of cellular damage than that incurred in the cortex. The relatively greater damage to cortical tissue is compatible with the previous observation in rabbits(2) that concentration of mercury in the cortex is approximately twice that in the medulla.

Summary. Sodium, potassium and water content of the renal cortex and medulla were determined in normal rabbits and in rabbits given intravenous injections of mercury, as mercaptomerin sodium, in doses of 0.5 to 20 mg/kg of body weight. Concentration of sodium in the normal medulla (103.1) meq/kg wet tissue) was considerably higher than that in the normal cortex (68.9 meq/kg). With administration of mercury in doses of 10 mg or more/kg, cortical sodium concentration and water content increased and potassium concentration decreased; medullary sodium and potassium concentrations both decreased.

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An Electrophoretic Study of Purified Thrombin.* (24833)

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In the present study, we investigated electrophoretic properties of purified bovine bioresin thrombin prepared as described by Seegers, Levine, and Shepard(1). The preparation had an average specific activity of 45,000 units/mg of tyrosine and 4100 units/mg of dry weight. In the analytical centrifuge the preparations showed a single symmetrical peak.

Materials and methods. Prothrombin was obtained from bovine plasma according to the methods of Seegers and co-workers(2,3). *Thrombin* was prepared by activating purified bovine prothrombin in the presence of lung thromboplastin and calcium ions(4). This material was then fractionated on the ion exchange column, the most active fractions were saved and finally obtained free of salt by methods previously described(1). Paper electrophoresis was performed with Spinco apparatus on Whatman No. 3 MM paper and with appropriate buffers of 0.1 ionic strength. Samples of .04% thrombin solution, .02 ml, were separated at room temperature using 120 volts for exactly 18 hours. The strips were then dried at 100°C and stained with 1% solution of bromphenol blue(5). After washing the strips in methanol, the color was intensified by ammonia fumes. The point of maximum color intensity was determined using a Photovolt densitometer. Samples of dextran,‡ .02 ml, were also included so that suitable corrections could be made for electro-osmosis(5). Staining of dextran by bromphenol blue was usually insensitive even after 3 hours in the dye. The dextran area finally appeared as a light blue band; staining with periodic acid-Schiff reagent confirmed its identity.

Results are depicted in Fig. 1. Electrophoresis was performed at pH 5.0, 5.5, 6.0, 7.0, and 8.6, using acetate, phosphate, and veronal buffers, of 0.1 ionic strength, in appropriate pH ranges. At completion of electrophoresis, the pH of buffer was again tested to insure that no changes had occurred. In nearly every experiment, a single peak was obtained. However, no inferences may be made with respect to electrophoretic homogeneity of thrombin preparation because of diffusion effects and the relatively poor resolving power of filter paper electrophoresis.

To determine mobility and isoelectric point, it was necessary to correct migration for degree of electro-osmotic flow of buffer toward the cathode. All measurements of migration by thrombin were made using the dextran area as the actual origin. Thrombin mobility was calculated(5) to be 3.14×10^{-5} cm^2/volt/second at pH 8.6 which compares with 3.02×10^{-5} cm^2/volt/second, the value for β-globulin obtained by Kunkel and Tiselius(5). In an experiment using a sample of human serum, the purified thrombin migrated to the same degree as the β-globulins.

The isoelectric point (Fig. 1) was 5.6.

Discussion. From previous measurements of electrophoretic mobility of thrombin, the following values were obtained: biothrombin, 6.5×10^{-5}; citrate thrombin, 4.6×10^{-5} and 6.0×10^{-5} cm^2/volt/second respectively for the 2 major peaks observed(6). Isoelectric points of 4.1 and 4.7 were determined for citrate thrombin(6). We believe that the difference between previous values and the ones reported here, namely mobility of 3.14×10^{-5} cm^2/volt/second and an isoelectric point of 5.6, is great enough to be significant. Therefore, when biothrombin, our source material, is subjected to ion exchange chromatography, the electrophoretic properties of the

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‡ Plavolex, a 6% solution of dextran, manufactured by R. K. Laros Co., Bethlehem, Pa.

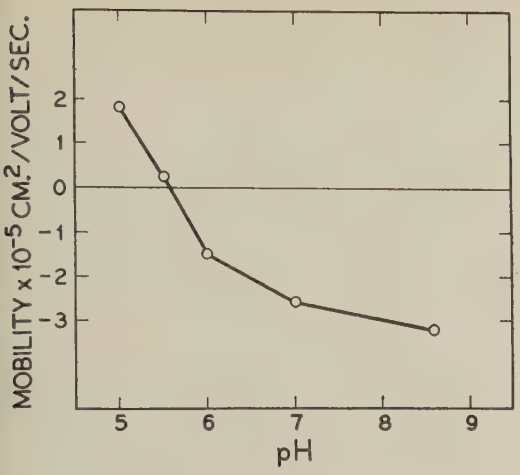

FIG. 1. Paper electrophoresis of purified thrombin with acetate, phosphate and veronal buffers, ionic strength 0.1.

material are altered as well as specific activity and ultracentrifugal properties already reported(1). In the citrate thrombin preparations previously studied it is likely that the results are in part the reflection of aggregate formation(7).

Summary. Electrophoresis on filter paper strips has been performed on purified thrombin prepared by recently developed technics of ion exchange chromatography. Mobility in veronal buffer, pH 8.6, ionic strength 0.1, was calculated to be 3.14 x 10^{-5} cm^2/volt/second. The isoelectric point was shown to be 5.6.

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Coupled Oxidative Phosphorylation in Crude Extracts of *Azotobacter agilis.* (24834)

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Oxidative phosphorylation in bacterial extracts is well authenticated(1-5), but details of this fundamental physiological mechanism are lacking. With exception of mycobacterial and corynebacterial systems of Brodie *et al.* (1), P/O ratios have been low compared to those theoretically possible, or are obtained with mammalian systems(6). It becomes desirable then to determine the influence, if any, of mode of preparation of the extract on P/O ratios. *Azotobacter agilis* (*A. vinelandii*) was employed as the test organism.

Materials and methods. Azotobacter agilis was grown with aeration in 3 L of Burk's nitrogen free mineral salts medium(7) containing 2% sucrose in 8-L pyrex glass bottle. The cells were harvested by centrifugation at 2°C and washed twice in cold distilled water. Intact cells, or disrupted ones, depending upon procedure employed, were suspended in 0.05 *M* "tris" (hydroxymethyl) aminomethane buffer, pH 8.0, containing 0.05 *M* $MgSO_4$; this solution is designated herein as TBS. Twenty-five μmoles ATP (N. B. Co.) were added to cell suspension or cell paste as stabilizer. *Sonic disintegration.* Two to 5 g of cells suspended in 5 ml of TBS were treated in 10 kc Raytheon magnetostrictive oscillator for 3 to 4 min. The homogenate was centrifuged 30 min at 20,000 X G and 2°C. The supernatant fluid (crude extract) was carefully decanted and enough phosphate buffer,

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pH 7.2, was added to give 15 to 20 μmoles of inorganic phosphate/ml. The same amount of phosphate was also employed in procedures to be described. *Grinding with alumina.* A quantity of cells (same weight as above but no fluid) was placed in mortar kept at 4°C. An equal weight of levigated alumina was added and the cells ground for 3 to 4 min at 4°C. After grinding, 5 ml of TBS were added and then centrifuged at 2,000 X G and 2°C for 10 min. The supernatant fluid was removed and recentrifuged at 2,000 X G and 2°C for 10 min before testing. Centrifugation at higher speeds removed active material. *Treatment in ball mill.* A quantity of cells equivalent to that used above was placed in a ball mill with equal weight of alumina and 15 stones and ground at 2°C for 30 min. Seven ml of TBS were then added. This material was centrifuged at 2,000 X G and 2°C for 10 min before determining activity of supernatant fluid. *Sudden release of pressure.* Nine to 13 g of cells (wet weight), suspended in 25 ml of TBS, were broken up according to method of French and Milner (see Colowick and Kaplan,8). The extract was centrifuged at 20,000 X G for 30 min at 2°C. Protein content of cell-free preparations was estimated by procedure of Stadtman *et al.*(9). Determinations of phosphate and manometric measurements were similar to those of Brodie and Gray(1). Since the extracts possessed no significant endogenous activity and there was little difference in effect of ADP added to dialyzed or non-dialyzed extracts, except to show stimulation by added acceptor with dialyzed extracts, dialysis of extracts was discontinued after first few experiments. Also, since dialyzed hexokinase showed no greater activity than undialyzed hexokinase, this treatment was also discontinued early in the investigation. Test systems consisted of 10 μmoles $MgCl_2$; 15-20 μmoles inorganic phosphate; 2 μmoles ADP (N.B. Co.); 20 μmoles mannose as the acceptor and 1 ml of crude extract in main compartment of Warburg vessels. The sidearms contained 50 μmoles of NaF, 50 μmoles of sodium succinate as substrate and 3 mg of yeast hexokinase (N.B. Co). The vessels were equilibrated for 10 min; then contents of sidearm were tipped in and oxygen uptake was measured 10 min. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid.

Results. Results recorded in Table I show that with crude extracts prepared by sonic disintegration P/O ratios are indeed low. In numerous experiments the ratios varied from 0.21 to 0.41, the highest consistent P/O ratios recorded in the literature for this organism with succinate as substrate being about 0.5 (3); and in one case, 0.8, with unwashed particles of Tissieres(5).

P/O ratios obtained under same conditions as above, but with extracts of alumina ground cells, were also low but equivalent to those observed with sonic extracts. Extracts prepared by using the French Cell also exhibited low P/O ratios, ranging from 0.15 to 0.33. Extracts prepared in ball mill were not as active oxidatively as those prepared by other methods, and were completely inactive phosphorolatively regardless of grinding time.

Time course study of oxidative phosphorylation showed, in a representative experiment, that with sonic extracts P/O ratio dropped from 0.57 at 2 min, to 0.36 at 5 min, decreasing to 0.28 at 10 min. In a representative experiment with French Cell extracts, P/O ratio dropped from 0.66 at 2 min, to 0.41 at 5 min, and to 0.25 at 10 min. These results suggest that a labile factor may be present in the extracts.

Centrifugal characteristics of extracts prepared by different methods are quite different, *e.g.,* a routine centrifugation of 20,000 X G for 30 min after breaking the cells by sonic disintegration or with French Cell, results in retention of activity in the supernatant fluid; however, centrifugation at 20,000 X G for 30 min, after breaking cells by grinding with alumina or in ball mill, separates almost all respective activities in the particulate fraction. This indicates a decided difference in size of particles obtained. Which are methodological artifacts and which truly represent the structure or structures of the intact cell await further clarification. Some measure of clarification has been offered by Bradfield(10), Tissieres *et al.*(5), and Cota-Robles *et al.*(11). In addition, the Table reveals an obvious difference with respect to specific activity (*i.e.,*

TABLE I. Oxidative Phosphorylation Ratios (μMoles Phosphate Esterified/μAtoms Oxygen Consumed) Observed with Crude Extracts of *Azotobacter agilis* under Conditions Described in Text.

System	Sonic	Alumina	Ball mill	French cell
Complete (duplicate)	2/7.5 = .26 2/7.4 = .27	4.2/10.2 = .41 3.4/10.2 = .33	0/3.6 0/3.3	1.6/10.6 = .15 1.8/10.4 = .17
No substrate	.3/ .8 = .38	1.2/ 1.1 = 1.09	0/0.3	.8/ .9 = .89
" acceptor	2/7.1 = .28	3.2/10.1 = .32	0/3.6	2.6/11.3 = .23
Neither substrate nor acceptor	0/ .5 = —	1 / .6 = 1.67	0/ .3	1.0/ .9 = 1.11
mg protein	9.5	7.8	7.4	16.8

activity/unit of protein) of different extracts.

It is clear from our results that the common methods of preparing extracts yield preparations exhibiting low P/O ratios with this organism. It is possible, in view of accumulating evidence, that there is a poor efficiency of coupling between endergonic and exergonic activities of *A. agilis*. In conformity with this view the extent of assimilation is low and this organism lacks sensitivity to dinitrophenol and azide both with respect to oxidative phosphorylation and oxidative assimilation(12).

Results reported in Table I indicate that in the absence of added substrate, oxygen consumption is markedly reduced, although in most cases P/O ratios are as high or higher than in complete systems. It is apparent that substrate oxidation is essential for fixation of larger amounts of phosphate, although added acceptor is not. Since ball mill extracts exhibited oxidative but not phosphorylative activity and quite marked oxidative ability was noted in all extracts, it appears that low P/O ratios may in part be the result of inhibition or destruction of part of the phosphorylating or phosphate transfer system. This is supported by decrease in P/O ratios with time observed in the extracts.

Summary. 1) The effect of method of preparation of the extract, sonic disintegration, grinding with alumina, disruption in ball mill, or sudden release of pressure in the French Cell, on P/O ratio of crude extracts of *A. agilis* with succinate as substrate has been investigated. 2) The P/O ratios show a wide range of variation with different extracts prepared by same method, but with all methods used, P/O ratios were low compared to mycobacterial and mammalian systems. Some evidence is presented that a labile factor is present in extracts and is responsible, at least in part, for the low ratios observed.

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Cholinergic Stimulation of Globus Pallidus in Man.* (24835)

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This report is concerned with effects of acetylcholine chloride, oxyphenonium bromide and sodium chloride injected into the region of globus pallidus of persons with Parkinson's disease.

Materials and methods. Eleven persons with varying degrees of Parkinsonism have been injected with cholinergic and anticholinergic drugs. Bilateral injections were done in 5 persons and unilateral in 6, 5 right and 1 left. Symptoms had been present from 3 to 30 years with increasing severity despite drug therapy. All antiparkinson medication was stopped prior to test period. The patient was alert and cooperative during injection periods, and numerous clinical tests were done but only 4 will be reported in detail. Motor strength in the hand was tested with bulb dynamometer (Geckeler type). Muscular rigidity was evaluated and observed by passively flexing and extending extremities at joints. Rapid alternating movements were tested in hand and fingers by having patient open and close the fingers as quickly as possible for 30 seconds. Differences in electrical resistance of skin over head, trunk and limbs were measured (A. R. Spartana Co. Model 2R Neuro-dermometer). Temperature of skin was recorded over same body areas with surface pyrometer (E. M. Rauh & Co., Inc.). A Cooper chemopallidectomy catheter without a balloon was placed into region of globus pallidus by free hand method. The position of tip of catheter was determined by measurement on the pneumoencephalogram. The ideal locus for the tip on lateral x-ray was 10 mm below and behind anterior lip of the Foramen of Monro, and on anteroposterior x-ray 15-20 mm lateral from midpoint of third ventricle. This would place the point of injection at medial third of globus pallidus. The catheter was securely anchored to scalp, and its position checked by x-ray before and after each injection period. Injection lasted 7-10 days, with average of 3 injections spaced several days apart. Acetylcholine chloride (Merck) in 10% solution and oxyphenonium bromide (Antrenyl, Ciba) in 0.2% solution were the drugs studied. Sterile isotonic saline and 5% sodium chloride were used as control injections. The drugs were dissolved in sterile isotonic saline at time of injection. Total amount of fluid injected was 1 ml and rate of injection was constant at 0.05 ml/minute. The patient sat up during injection to prevent regurgitation of fluid along catheter track.

Results. A total of 88 injections has been done, 56 with acetylcholine chloride, 16 oxyphenonium bromide, 15 isotonic saline, and 1 with 5% saline. The events reported with each drug did not necessarily occur following every injection but represent a composite of observations.

Isotonic saline and 5% saline solution resulted in no clinical change.

Acetylcholine chloride. During initial 5-10 minutes of injection, a stimulatory effect occurred with increase in amplitude of tremor in the contralateral arm or onset of focal seizure in the contralateral face. Conjugate deviation of eyes or turning of head away from injected side was noted. Increase in tremor was most often unilateral, but in one man the tremor increased in both arms, but more so in the contralateral extremity. Increase in tremor lasted only a minute or 2 while focal twitches or adversive eye movements might continue several hours. The lower extremity was not involved in this increased activity even though tremor and rigidity were present. During this period the patient was conscious. Rigidity and tremor were reduced after initial excitatory stage; the patient would exclaim about looseness of the contralateral upper limb. The arm was most often involved with less noticeable effect in the leg. An increase in rapid alternating movements and range of movement accompanied this decrease in rigidity. Reduction was most pronounced in forearm, wrist and fingers. Loss of rigidity occurred in

* Work supported by Parkinson Foundation, Inc.

some instances without any motor weakness. Following a single injection, rigidity decreased for several hours to days. Hoffman or Babinski sign was noted in several persons within a few minutes of injection. The reflex change was not necessarily found in the extremity with reduction of rigidity or tremor. The reflex change lasted several hours or a few days when it disappeared but would then reappear with repeat injection. Parasympathetic stimulation occurred at time of injection with increased sweating in contralateral arm or leg, and a rise in skin temperature. Nausea and vomiting occurred in only a few instances, for a short time. Two persons became unresponsive during the first few minutes of injection. This was not associated with fall in blood pressure but there was a drenching sweat, and one person developed extrasystoles. Within 5 minutes both were awake and normal. Blood pressure, pulse and respirations rarely changed during injection.

Oxyphenonium bromide. Rigidity was reduced in 5 instances, tremor in 2, with no change in motor power. The sudoresis on opposite side of body produced by intracerebral acetylcholine was blocked by intracerebral injection of oxyphenonium bromide. A central facial paresis was noted after 1 injection, a Babinski reflex once. One man had difficulty opening his eyes after injection. One man became sleepy and exclaimed that his head had gone to sleep. Nausea and vomiting did not occur. Several persons complained of dryness of mouth 15-30 minutes after injection, and a blotchy erythema occurred in one man.

Discussion. Cushing(1) demonstrated parasympathetic stimulation of the hypothalamus by intraventricular injections of pilocarpine in man. Henderson(2) produced similar parasympathetic responses following intraventricular injections of acetylcholine and eserine. Our report indicates that parasympathetic effects may occur on contralateral half of body after injection of a cholinergic drug into the region of globus pallidus. The site of this stimulation is not certain as diffusion into the adjacent internal capsule and/or hypothalamus may occur. It is possible to block this response by intracerebral injection of oxyphenonium bromide into the same region. Electrical and mechanical stimulation of the lateral wall of 3rd ventricle in man(3) and animals(4) may result in altered consciousness and cardiac arrhythmia. Two patients reported here had an intense parasympathetic response, unconsciousness and cardiac arrhythmia, suggesting chemical stimulation of the hypothalamus.

The mechanism of drug action on rigidity and tremor is not clear from these observations. Alterations in vascularity in the injected region may be responsible. A purely mechanical effect caused by injecting a fluid seems unlikely in view of negative results with saline injections. Concentration of acetylcholine was high, and it is well known that changes in concentration of a drug may result in excitatory or depressive effects. It is possible that high concentration resulted in a block of nervous activity in the region of globus pallidus. Bailey(5) has demonstrated a blocking effect of procaine hydrochloride in frontal lobes of man, and Narabayshi(6) postulated the blocking effect of procaine in the globus pallidus as responsible for reduction of tremor and rigidity in his cases of Parkinsonism.

Summary. A 10% solution of acetylcholine chloride injected into the globus pallidus of persons with Parkinsonism resulted in initial excitatory stage with increase in tremor and parasympathetic activation. Parasympathetic response was blocked by intracerebral injection of an anticholinergic drug, oxyphenonium bromide. The exact mechanism of drug action is not known, although cholinergic sensitive regions appear to exist in or near the globus pallidus.

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Fractions with High Activity for Intrinsic Factor and Combining Vit. B_{12} With Receptor Substances.* (24836)

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Although attempts have been made to isolate intrinsic factor (IF) from hog mucosal extracts in pure form, no homogeneous preparations have apparently been obtained(1). One of the principal difficulties has been the absence of a rapid *in vitro* assay for intrinsic factor activity. Miller and Hunter(2), reported that uptake of Vit. B_{12} by liver slices was stimulated by IF concentrates and suggested that it could be used as an assay for IF. This method was further developed by Herbert(3). Miller(4) and Miller and Hunter(5) have also reported that IF concentrates greatly increase the combination of B_{12} with receptor substances prepared from serum and liver tissue and proposed that this reaction be employed as an assay for IF activity. Cellulose-ion exchangers described by Peterson and Sober(6) have been used successfully to isolate proteins of blood serum (7) and egg white(8). The cellulose cation-exchanger, carboxymethylcellulose (CM-cellulose), has been employed in the present study for isolating fractions from hog stomach mucosal extracts which had high activity for increasing the combining of B_{12} by the receptor substances of Miller and Hunter(5) in an *in vitro* assay.† These fractions have also been found to have activity for IF by the Schilling technic in humans.

Methods. Starting materials with IF activity were extracts of hog stomach. Both initial aqueous extract and dry concentrate prepared therefrom were employed (Wilson Labs., Chicago, Ill.). Dried concentrate was so prepared that 10 mg when combined with 10 μg of Vit. B_{12} constitutes 1 U.S.P. (oral) unit. Material was prepared by dialysis against buffer of 0.1 *M* acetic acid titrated to desired pH with NH_4OH. Insoluble precipitate was removed, washed and washings combined with supernatant. CM-cellulose, containing 0.6 meq. of titratable acidity/g, was prepared by method of Peterson and Sober(6). Use of the exchanger was essentially similar to that previously described for the fractionation of egg white(8). All fractionations were carried out at room temperature. Assays for IF activity on humans were done by the Schilling technic (9). In this assay preparations required in the least amount have highest activities. Results of *in vitro* assays(5) are given in terms of radioactive counts/min/mg of preparation tested. The preparations which give highest counts have highest activities. Estimations of protein concentrations were routinely obtained by determination of absorbances at 280 mμ with a Beckman Model DU spectrophotometer. Sialic acid was determined by the method of Werner and Odin(10) except that sulfuric acid was employed rather than hydrochloric acid in the direct Ehrlich's test. The sialic acid used for standard was prepared from egg white. It had properties similar to crystalline material prepared from *Escherichia coli*.‡ Paper electrophoretic analyses were performed with horizontal strip apparatus using potassium phosphate, 0.1 μ, pH 6.9, as buffer. A constant current (350 volts; 8 milliamperes) was used for 18-24 hours. Moving boundary electrophoretic analyses were performed with American Instrument Co. portable electrophoresis apparatus.

Results. I. *Preliminary fractionation.* Approximate conditions required for initial adsorption and elution of fractions with *in vitro* activity were initially established.

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‡ This preparation of sialic acid was kindly supplied by Dr. Saul Roseman and Dr. D. G. Comb, Univ. of Michigan.

TABLE I. Fractionation of IF Concentrate on Carboxymethylcellulose.

Description	Fraction	Vol, ml	pH of eluate	Dry, mg	*In vitro* assay, count /min./mg $\times 10^3$	Total activity, count/min. $\times 10^6$
Initial fractionation	IF conc.			20,000	50	1,000
	A-1*	1200		3,960	111	438
	2	1450	3.9	1,885	90	170
	3	1115	5.0	780	344	268
Refractionation	3	1064		744	344	256
	4	1380	3.9	230†	9	<2
	5	165	4.0	20†	50	<1
	6	50	4.0–4.4	37	2,622	97
	7	175	4.4	40	925	37
	8	68	4.4–4.6	58	672	39
	9	38‡	4.6	46	457	21
	10	195	4.6–7.0	585†	48	28
Starting extract				3,795		438
1st fractionation (A-2 and A-3)				2,665		438
Material refractionated A-3				744		256
Refractionation (A-6 to A-9)				181		194

* A-1 was initial extract from IF concentrate. 1150 ml used for fractionation.
† Estimated from absorbance at 280 mμ.
‡ Concentrated from 423 ml by adsorption at pH 4.0 on 3 g CM-cellulose and elution at pH 9.0.

II. *Principal fractionations.* In experiment presented in Table I, 20 g of powdered concentrate were fractionated. The soluble and dialyzed extract contained 20.8% of original dry weight and 43.8% of total original apparent activity. This extract (A-1) of dry concentrate was mixed with 15 g of CM-cellulose at pH 3.9 and filtered and washed on a Buchner funnel. The combined filtrate and washings were the pH 3.9 fraction, (A-2). The exchanger cake was resuspended in buffer at pH 5.0, adjusted with NH_4OH, then filtered. The cake was washed with pH 5.0 buffer, combined eluate and washings (A-3) redialyzed at pH 3.9 and refractionated through a column of 3 g of CM-cellulose. Fractions A-4 to A-10, obtained by stepwise elution on a fraction collector, were dialyzed and activities determined by the *in vitro* method. As is apparent, the most active fraction emerged from the column between pH 4.0 and 4.4 with decreasing amounts between pH 4.4 and 4.6. Three-fourths of the total activity put on column emerged in pH interval of 4.0 and 4.6 but less than a quarter of dry weight was in this fraction.

In Table II, results are given of fractionation of 40 g of dry concentrate. In this instance, the extract (B-1) was passed through 40 g of CM-cellulose as a compacted cake on a Buchner funnel under slight vacuum. Fractions B-2 to B-11 were obtained by step-wise changes in eluting buffer. Approximately 100 ml fractions were removed manually and absorbance read at 280 mμ. As in the previous fractionation, the activity peaked at pH range of 4.2 to 4.4 with decreasing amounts to pH 5.0. In this fractionation, 95% of activity put on the exchanger was recovered in the pH 4.1 to 5.0 interval but only 10% of dry weight.

Five other fractionations were also carried out on various amounts of the dry concentrate (2 to 20 g). In complete agreement with above described runs, peaks of activity by *in vitro* assay were obtained between pH 4.1 and 4.4 with decreasing amounts to pH 5.0. Specific activity of peak fractions varied from 7.5×10^5 to 2.01×10^6 counts/min/mg. Likewise, approximately 75% of activity and 25% of solids placed on the column were recovered in fractions eluted from pH 4.1 to 5.0.

III. *Direct isolation from aqueous extract.* Two fractionations were made starting with aqueous intrinsic factor extracts. The first fractionation of 300 ml of dialyzed extract was made on a 3 g column of CM-cellulose starting at pH 4.0 and eluting in a stepwise fashion. Again the active fraction by *in vitro* as-

TABLE II. Fractionation of IF Concentrate on Carboxymethylcellulose Employing Buchner Funnel.

Fraction	Vol, ml	Elution pH	Dry, mg	*In vitro* assay, count /min./mg $\times 10^3$	Total activity, count/min. $\times 10^6$
IF conc.			40,000	50	2,000
B-1*	1700		11,000†	67	740
2	1815	3.9	2,568	11	27
3	350	3.9	252	40	10
4	775	3.9–4.1	434	168	73
5	350	4.1–4.2	147	272	40
6‡	995	4.2–4.4	239	1,201	287
7	700	4.4	119	487	58
8	48§	4.4	57	368	21
9	1515	4.4–4.7	258	531	137
10	1100	4.7–5.0	317	511	162
11	1500	5.0–6.0	1,830	56	102
Material on column			11,000		740
" eluted, pH 3.9–6.0			6,221		917
" " , " 4.1–5.0			1,137		705

* B-1 was initial extract from IF concentrate.
† Estimated from absorbance at 280 mμ.
‡ Fractionation interrupted overnight after this fraction before the next fraction was obtained.
§ Concentrated from 964 ml by adsorption on 4 g. CM-cellulose at pH 4 and elution at pH 9.

say occurred between pH 4.0 and 4.5. Specific activity at peak was 4.96 X 10^5 counts/min/mg. A second isolation of intrinsic factor was made from undialyzed extract. One l of the extract was centrifuged to remove insoluble precipitate and the supernatant was adjusted to pH 4.7 and passed through a compacted cake of CM-cellulose (50 g) on a Buchner funnel. The eluate was adjusted to pH 4.2 with 0.1 *M* acetic acid and water added to maintain the ionic strength of buffer. This was passed through a second 50 g cake of exchanger on Buchner funnel. After washing exchanger cake with pH 4.2 buffer, the active fraction was eluted at pH 9.0. (Elution was done with buffer of high pH, to obtain a rapid elution of the protein). This eluate was refractionated as above with the exception that 12 g cakes of exchanger were used. The yield obtained was 148 mg. An *in vitro* assay was not obtained on this sample (G-1); however, several human assays were performed (Table III).

IV. *Comparison of activities by human and in vitro assays.* A summary of results obtained in comparison of activities of different fractions by *in vitro* and human assays is given in Table III. The peak of activity by *in vitro* assay appeared at pH 4.3 to 4.4. However, most active preparations by Schilling assay were obtained at a slightly higher pH range, 4.4 to 4.6.

V. *Chemical and physical properties.* Be-

TABLE III. Comparison of Activities by *In Vitro* and Schilling Assay.

Fraction	pH of eluate	*In vitro* assay, count/min./mg $\times 10^3$	Schilling* assay	
			Active	Dose, mg
A-6	4.0–4.4	2,622	No	2.0
7	4.4	925	Yes	.56
8	4.4–4.6	672	"	.75
9	4.6	457	"	1.2
B-9	4.4–4.7	531	"	.75
B-9 + 10†	4.4–5.0	521	"	.88
C-1	4.4–4.8	650	"	.70
D-1	4.1–4.4	1,250	No	.60
E-1	4.1–4.3	2,000	"	.60
E-2	4.3–4.5	1,000	Yes	.56
F-1	4.3–4.5	894	"	.55
G-1	4.2–4.7		"	1.0

* An active fraction was that which gave at least half maximum urinary excretion of standard dose of radioactive B_{12}. The minimum amount to give this is believed to be approximately equivalent to daily oral dose for pernicious anemia patient in relapse (= 1 U.S.P. unit). Dosage for the active samples as the lowest tested and may not be the minimum amount needed for activity. Likewise, dosage for the inactive samples was the maximum tested. Larger doses of these samples might show activity. Figures are actual weights of doses.
† An equal aliquot mixture of these 2 fractions.

cause of current interest in sialic acid and its presence in mucoprotein, analyses for sialic acid were run on preparations listed in Table III. Sialic acid contents varied from 2.2 to 3.4% on dry weight basis. Paper electrophoretic analyses of fractions A-6, A-7, and A-8 indicated the latter was essentially one component when run in phosphate 0.1 μ pH 6.9, while A-6 and A-7 gave one major component and only a trace of a second. A moving boundary electrophoretic analysis was run on 0.5% solution of preparation E-2 in 0.1 *M* sodium acetate, pH 4.4 at 1°C for 21,300 sec. with potential gradient of 4.2 volt/cm. Under these conditions, only one component was evident and had a very low negative mobility. Both the electrophoretic analyses and the pH of elution from CM-cellulose indicate an approximate pK (or isoelectric point) of pH 4.5 for the material with intrinsic factor activity by the Schilling assay.

Discussion. Replicate fractionations of dry intrinsic factor concentrates and of aqueous extracts under similar conditions have all yielded very active intrinsic factor preparations. This reproducibility was unexpected in view of published report where DEAE-cellulose was employed(1). The reproducibility obtained in present studies was similar to that obtained when CM-cellulose was employed to fractionate egg white(8).

Further study is necessary for interpretation of apparent difference in optimal pH for elution of activity, as determined by *in vitro* assay and human assay. This difference is so small (less than 0.2 pH unit) that more assays are necessary to prove or disprove heterogeneity. It is possible that the difference is due to presence of an activator or inhibitor in one fraction which affects one assay but not the other or that there are 2 substances. These might differ, however, in only a very minor way such as presence of an additional acidic or basic group. A separation of a substance with 2 such closely related structures has been reported in ovalbumin from egg white. Ovalbumin A_1 and ovalbumin A_2, which differ by one phosphate group, were separated on CM-cellulose at an interval of less than 0.2 of a pH unit(8).

Summary. Extracts of hog stomach mucosa were fractionated on cellulose-cation exchanger, carboxymethylcellulose. Highest activity for increasing the combining of Vit. B_{12} with receptor substances of tissue was eluted between pH 4.2 and pH 4.4. Highest activity for intrinsic factor by the Schilling technic was eluted between pH 4.4 and pH 4.7. An approximate pK (or isoelectric point) of pH 4.5 was indicated for the material with intrinsic factor activity by the Schilling assay. These fractions contained 2 to 3% sialic acid.

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Responses of Swine to High Doses of Radiation. (24837)

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Small laboratory animals have been studied extensively at high radiation doses(1,2) and a few results with monkeys(1), dogs(3) and swine(4) have been reported. Because of some striking differences in response patterns of different species more data are desirable. Large animals having cross sections comparable to that of man are of particular interest since similar depth dose distributions can be obtained. Laboratory studies at high doses have been limited by the requirement for high dose rate over a large field, so that exposure times will be short compared to survival times. With high intensity X-ray source available, high dose studies at reasonable rates became possible in mammals as large as swine, which have been utilized in this study. In addition to adding detailed information on another species, use of swine provided better data on high-dose incapacitation than is obtainable from smaller animals.

Methods. Twenty-six cross-bred swine 3- to 4-months-old, weighing from 80 to 130 lb, were used. Pre-irradiation hemograms and complete physical examinations were done on all swine, and all were in good health at time of irradiation. The X-ray source and calibration procedures have been described(2). Swine irradiations were done at 3.0 to 3.5 Mev constant potential beam having an HVL of 21 cm in masonite phantom. With target-skin distance of 180 cm, dose rate in air at mid-swine position was 150 r/min. Special filters gave a radiation field flat to $\pm$ 5% to a radius of 2½ ft. which permitted simultaneous exposure to 4 swine. At highest doses, exposure times were an appreciable fraction of survival times calculated from the mid-point of irradiation. Behavior during irradiation was observed with a closed-circuit television system. After irradiation all animals were under constant observation until death. Blood samples were collected from anterior vena cavae for hematological studies by standard methods. Properdin titers of sera of some swine were determined.* Sections of stomach and intestines were removed immediately after death and complete autopsies performed within 2 hours. Tissues were examined grossly and microscopically.

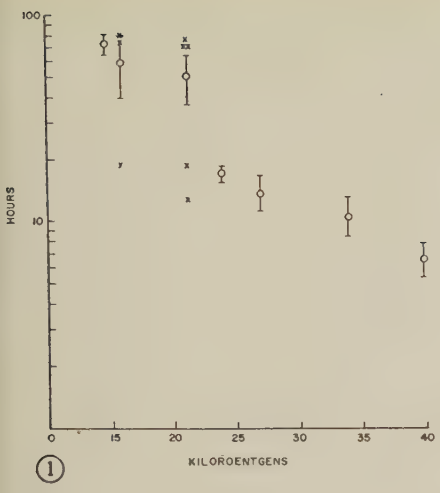
Results. At this dose rate the first sign of radiation injury was restlessness, starting at 2000 to 2500 r and increasing with dose. At 3500 to 4000 r (22 to 27 minutes) acute distress was evidenced by extreme salivation, mastication, retching and profuse emesis. Diarrhea appeared at about 15,000 r. With exception of diarrhea, these manifestations ceased at 10,000 to 20,000 r and were followed by calm which progressed to a marked depression.

Although depressed, the swine recognized auditory and visual stimuli after 20,000 r (133 minutes) and were capable of responding to them. Depression continued to increase with dose, however, and by 30,000 r (200 minutes) all swine were prostrate. They did not respond to auditory, visual or dermal stimuli. The conjunctival reflex was absent and ataxia was evident when the swine attempted to rise. Transient central nervous system manifestations appeared at 39,000 r (260 minutes) with convulsions and loss of equilibrium.

Swine normally have a rectal temperature of 101.6 to 103.6°F, pulse 60 to 80/min and respiratory rate of 8 to 18/min. By 14,900 r mean values had reached 105.0 $\pm$ 0.2°F, 169 $\pm$ 26/min and 67 $\pm$ 17/min. At this stage respirations were irregular with prolonged expiratory phase. Respiration became increasingly rapid and shallow as dose progressed.

At doses of 21,350 r and below, the swine were markedly depressed and moderately ataxic when exposure was terminated. Some presented diarrhea and vomiting. Of interest was their striking improvement from 1 to

* Courtesy of Dept. of Serology, Walter Reed Army Inst. of Research.



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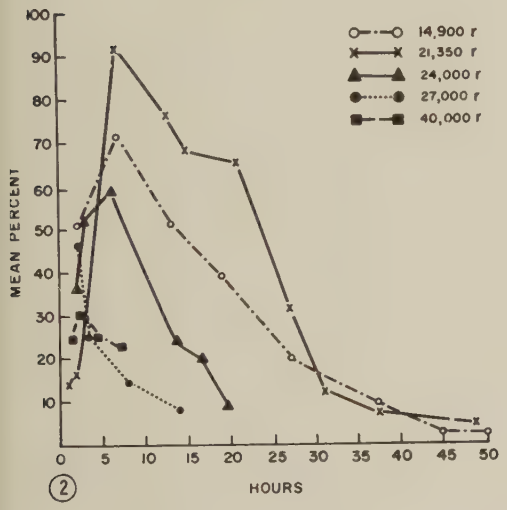

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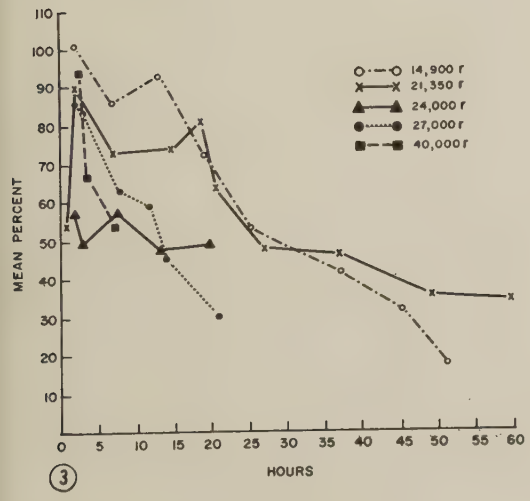

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3 hours after exposure. Even though diarrhea and vomiting persisted, several ate, drank small amounts and foraged through the bedding. It appeared that at least simple, and probably more complicated, tasks could have been performed. This period of "well-being" persisted 10 to 48 hours in various animals, after which their condition deteriorated. Thereafter, death ensued within a few to 24 hours. The 24,000 and 27,000 r groups presented minimal recovery and were more like higher dose groups in their response.

The highest dose swine (40,000 r) were completely incapacitated after exposure. Mean rectal temperature was 106.6 $\pm$ 0.3°F, pulse rate 172 $\pm$ 11/min, and respiration 84 $\pm$ 23/min. They were prostrate and even when assisted could not stand. Nystagmus developed within an hour or 2 and pupillary response to light was considerably diminished. Transient seizures appeared from 10 minutes to 3 hours after completion of exposure. These progressed from transient episodes of clonic spasms of various muscle groups and false "running" movements to severe convulsions and opisthotonos. Pulse rate remained approximately twice normal, respiratory rate 4 to 5 times normal and temperature response varied throughout the group. The swine remained completely incapacitated. Death occurred during violent convulsions. The 34,000 r group was similar in response.

Fig. 1 presents mean survival time by dose group. Standard errors are indicated. The wide limits at 16,000 r and at 21,350 r result from a definite "split" in survival time, *i.e.*, these swine either died in less than 20 hours or survived more than 70 hours. Individual survival times at these doses are given in Fig. 1.

Mean leukocyte responses are presented in Fig. 2. For clarity in the Figure, responses

FIG. 1. Survival times of swine exposed to various supralethal doses of X-radiation. Mean and stand. error of mean are indicated for each dose. Individual points plotted for 2 doses due to definite "split" in survival times.

FIG. 2. Circulating leukocyte response of supralethally irradiated swine at various times after exposure. Plotted points are mean percents of mean pre-irradiation values.

FIG. 3. Blood platelet response in swine at various times after supralethal irradiation. Points are mean percents of pre-irradiation mean values.

at 16,000 r and at 34,000 r are omitted since they closely resembled those at 14,900 r and 27,000 r respectively. In all, swine lymphocytes were drastically reduced on first post-irradiation sample and were at minimal levels by the 12-hour sampling. The relative proportion of segmented neutrophils was increased immediately after irradiation as was the absolute count. This rapidly changed to a predominance of band forms. Myelocytes and metamyelocytes were present immediately after irradiation and persisted about 36 hours or until death. In many swine normoblasts were present immediately post-irradiation. Though decreased in numbers these persisted for as long as 12 hours. After about 36 hours, eosinophils became the predominant cell type. Many of these were eosinophilic metamyelocytes. In some swine toxic granulation of neutrophils appeared as early as 26 hours. Those swine surviving more than 70 hours presented a profusion of basket cells prior to death.

Mean blood platelet responses are presented in Fig. 3. As in Fig. 2 16,000 r and 34,000 r groups are omitted.

Erythrocyte counts of only the 14,900 r group decreased at 36 hours post-irradiation (16,000 r unsatisfactory). Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration values were not significantly different after irradiation. Before irradiation erythrocyte fragility values were 0.608 $\pm$ 0.0058 and 0.433 $\pm$ 0.0042 for partial and complete hemolysis respectively. At all times after irradiation the 34,000 r swine (40,000 r not done) presented significantly increased fragility. Mean value for partial hemolysis was at least 0.690 (endpoint missed in some samples) and was 0.493 $\pm$ 0.0053 for complete hemolysis. By 8 hours after 27,000 r respective mean values for partial and complete hemolysis were at least 0.651 (end-point missed) and 0.461 $\pm$ 0.0126. This increased fragility persisted until death. Other dose groups did not present significantly altered erythrocyte fragility. In view of exposure time reticulocyte (proerythrocyte) values are considered for only the 3 lower dose groups. Based on mid-point of sampling interval, these disappeared at 24 hours in the 21,350 r and the 16,000 r groups and were absent by 34 hours in the 14,900 r group.

Hematocrit levels decreased after irradiation although the changes were not statistically significant. In most cases terminal samples presented hematocrit values much higher than normal. The uncorrected erythrocyte sedimentation rate increased drastically and continuously from 24 hours after irradiation until death. Not infrequently these values were in the range of 45 to 55 mm.

The properdin response in blood serum in the 14,900 r, 24,000 r, 27,000 r and 34,000 r groups was characterized by mean decrease of 32% from 12 hours onward. If all samples on all swine for 12 hours and more after irradiation are combined, a mean of 4.8 units/ml, as compared with pre-irradiation mean of 7.1 units/ml, results. This difference is statistically highly significant ($p \leq 0.005$).

Discussion. Although the numbers of swine are not large, these data suggest an abrupt survival time transition as in the guinea pig or mouse, rather than the smooth semi-log response of rat and hamster. This is borne out by the shift from depression to convulsions and to some extent by blood data. The abrupt transition in mean survival time between the 21,350 r and 24,000 r groups with an exponential relationship at doses above the transition zone undoubtedly reflects a different mode of death. Although occurring at a point intermediate between those for guinea pig (6 Kr) and mouse (43 Kr), it is unlikely that the phenomenon is different.

The lower dose rate can probably be considered as responsible for only slight evidence of Langham *et al.*'s acute ataxic phase(1). Their lethargic, hyperactive and terminal phases were in evidence. Even more similar was the clinical response of guinea pigs(5,6). At doses above 30,000 r, swine remained completely incapacitated throughout the brief post-irradiation period. It appeared that the minimal recovery at 24,000 r and 27,000 r would not have allowed performance of other than the most basic tasks, if any. Following a period of 1 to 3 hours after irradiation, swine receiving lower doses presented dramatic recovery and undoubtedly could have func-

tioned with only partial impairment until 10 to 48 hours after irradiation. During exposure, accumulation of about 4,000 r greatly impaired function as a result of nausea and severe emesis.

These and previously cited data emphasize the differences in species and leave the response of man unknown. It may be of significance that in Japan there was no mention of an excitatory phase but depression was of common occurrence. Practically, it may make little difference, for radiation depressed humans may be able to function for varying periods because of cerebral motivation questionably present in animals. Further, man may be reasonably functional between convulsions. Thus, evaluation of incapacitation in these swine may not be indicative of that occurring in heavily irradiated human population.

At this dose rate granulocytosis was not observed immediately post-irradiation. Transient leukocyte recovery at lower doses was undoubtedly due to release of immature forms into the circulating blood. The presence of numerous basket cells by the third day suggests that destruction of the mechanism for removal of damaged neutrophils may occur at doses lower than previously reported(1).

Platelet counts start to decline about the third or fourth day after mid-lethal range irradiation. Since decreases were initiated much earlier in these swine, damage to platelet forming and release mechanisms must have been more profound. These responses suggest dose dependency.

Increased fragility of erythrocytes in the 27,000 and the 34,000 r swine demonstrates a decreased resistance to hemolysis after massive doses of irradiation. Osmotic fragility of young mature human erythrocytes is less than that of older cells(7). Even if the same is true for swine, it is difficult to conclude that the cell population studied was so largely composed of older erythrocytes since increased fragility was present soon after irradiation. It seems unlikely that increased fragility can be attributed to a greater radiation susceptibility of younger erythrocytes; thus, the mechanism remains in doubt. The slight decrease in hematocrit may be indicative of erythrocyte destruction. The terminal increase in hematocrit was undoubtedly due to hemoconcentration.

Significance of the properdin system in the response to mid-lethal irradiation remains in doubt(8,9). In swine reported herein, a decrease occurred earlier than for mid-lethal irradiation in other species, but did not continue. Relation to survival time could not be demonstrated. The significance of the decline is difficult to assess.

The gross and microscopic pathological changes will be reported elsewhere.

Summary. Although time and dose relationships were different, the response of swine to high doses of radiation was similar to that of guinea pigs and mice. For practical purposes, at doses of 24,000 r and above, incapacitation was complete in these swine. It is conceivable that if these animals possessed cerebral motivation similar to that in man, capabilities might have been greater. At lower doses a striking recovery occurred within 1 to 3 hours after exposure.

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Activation of Venom Proteases and Reversal of Chelating Effects by Sodium Bicarbonate.* (24838)

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Proteolytic activity of rattlesnake venom on gelatin layer of photographic film is activated by mammalian serum and its ash(1). Attempts to achieve the same degree of activation by specific inorganic substance were previously unsuccessful, although salts containing phosphate ion brought about partial activation. In the present work the bicarbonate ion in concentration approximating that in mammalian serum had the same activating effect as whole serum on rattlesnake venom gelatinase. This ion also reverses the effect of chelating agents on both water moccasin and rattlesnake venom gelatinases and increases proteolytic activity of these venoms on urea denatured hemoglobin.

Materials and methods. Venoms of the water moccasin (*Agkistrodon piscivorus* and Eastern diamond back rattlesnake (*Crotalus adamanteus*) were purchased from Ross Allen's Reptile Inst. in dry powdered form. They were dissolved in 0.9% sodium chloride solution and kept in frozen state until immediately before use. Inorganic compounds and chelating agents were of analytical reagent grade. Solutions of sodium bicarbonate were prepared initially in 0.02N concentration and pH adjusted to 7.4 with hydrochloric acid. The photographic film was Eastman's Kodak verichrome panchromatic. Urea denatured hemoglobin was prepared using modification of method by Anson(2). After urea and sodium hydroxide had been added to the dialyzed beef erythrocytes, the mixture was allowed to stand for one hour at room temperature, then dialyzed 24 hours against running tap water and another 24 hours against several baths of distilled water. Dialysis removed all urea and brought the pH from 12.0 to 7.7. Some of the hemoglobin precipitated, but enough remained in solution to maintain a concentration of 0.8 g%. Enough sodium chloride was added to this solution to bring the concentration to 0.9% and hydrochloric acid was then added to bring the pH to 6.8. Sodium bicarbonate, disodium phosphate, and sodium tetraborate (borax) were then added to aliquots of this material as indicated in Table II (0.02N concentration) and pH of all solutions was adjusted to 7.4 $\pm$ 0.1 by addition of HCl. Sodium hydroxide was added to a fourth aliquot of hemoglobin (pH 6.8) to bring the pH to 7.4. The digestion of gelatin layer of photographic film was carried out by method previously described(1). Doubling dilutions of the venom were prepared in 0.1 ml quantity with and without various activators and inhibitors in 0.1 ml quantity in concentrations shown in Table I. Two-hundredths (0.02) ml of each solution was placed on ¼ inch strips of film, and after 1 hour incubation at 37°C, the film strips were rinsed in gently flowing tap water. Figures in Table I represent smallest amount of venom in μg required to completely remove gelatin from the nitrocellulose base of film. Although there is an analytical error of 50 to 100%, activation of 2500% is reported. Variations of 100% or less, such as slight decrease in activity of moccasin venom by DHEG (line 11, Table I) are not considered significant. Digestion of hemoglobin was carried out by the method of Anson(2). Venom solutions with various anions were added to urea denatured hemoglobin and incubation carried out at 37°C for 15 minutes. The amount of tyrosine liberated was determined by use of Folin-Ciocalteau reagent. Disodium phosphate, sodium bicarbonate, and sodium tetraborate have a buffering action at pH 7.4 and of these sodium tetraborate is the most effective. The hemoglobin also has some buffering effect. Water moccasin venom toxicity studies were carried out in mice by mixing maximum tolerated dose of various inhibitors with 2-3 LD_{50}'s of venom and inoculating with these solutions intra-

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TABLE I. Effect of Various Substances on Amount of Venom Required for Liquefaction of Gelatin on Film.

	Venom	
	Water moccasin*	Rattlesnake*
Saline control (pH 7.4 with $Na_2B_4O_7$)	2	25
Substances added†		
Human serum	1	1
Acid ash		25
$NaHCO_3$ (.02 M, pH 7.4 with HCl)	1	1
Acidified $NaHCO_3$		25
Na_2HPO_4 (.02 M, pH 7.4 with HCl)		6
Penicillamine (.6-1.0%, pH 7.4 with NaOH)	50	100
Penicillamine (.6-1.0%, pH 7.4 with $NaHCO_3$)	2	12
EDTA (2.5×10^{-3} M, pH 7.4 with NaOH)	33	67
EDTA (2.5×10^{-3} M, pH 7.4 with $NaHCO_3$)	8	17
DHEG (.1 M, pH 7.4 with NaOH)	4	25

* μg of venom in .02 ml required to remove gelatin from film in 1 hr at 37°C and 100% moisture.
† .1 ml of activators and inhibitors in concentrations listed plus .1 ml of venom dilutions in .9% sodium chloride sol.

peritoneally. The effect of these inhibitors on rattlesnake venom was determined by inoculating with the chelating agent intraperitoneally prior to inoculation with the venom. Observations on mortality were made for 48 hours. Acid ash of human serum was prepared by bringing the pH of an aqueous solution of ash to 1.5 with hydrochloric acid, boiling, and readjusting pH to 7.4 with sodium hydroxide. A solution of sodium bicarbonate initially .02N concentration was acidified in like manner with hydrochloric acid and restored to pH 7.4 with sodium hydroxide.

Results. Rattlesnake and water moccasin venom gelatinases in unbuffered saline are not affected by pH changes within range of 5 to 8. Sodium tetraborate has no effect on activity of these enzymes and is therefore the compound used to adjust the pH of saline controls to 7.4.

As shown in line 1 of Table I, 25 μg of rattlesnake venom in borax buffered saline are required to liquefy gelatin whereas only 1 μg is required if sodium bicarbonate is added. This 25-fold activation is equal to that of human serum and its ash and is 4 times greater than that of sodium phosphate. Acid ash of human serum and acidified sodium bicarbonate have no activating action (Table I). Ethylenediamine-tetraacetic acid (EDTA) and penicillamine are effective inhibitors of water moccasin venom gelatinase when the pH of these compounds is brought to 7.4 with sodium hydroxide. When sodium bicarbonate is used to adjust the pH, the inhibitory action of these compounds is diminished. Similar results were obtained using these chelating agents on rattlesnake venom although the inhibitory effect of EDTA is slight. The iron chelating agent dihydroxyethyglycine (DHEG) has no significant effect on venom proteolysis at pH 7.4

When urea denatured hemoglobin is used as substrate (Table II), 1 mg of water moccasin venom without sodium bicarbonate will liberate 3.8-4.0 millimoles of tyrosine X 10^{-4} in 15 minutes at 37°C and with bicarbonate the amount is increased to 6.4 mM X 10^{-4}, an increase of 70%. Two mg of rattlesnake venom liberate 0.9 to 1.0 mM of tyrosine X 10^{-4} in the absence of sodium bicarbonate and 2 mM X 10^{-4} with bicarbonate, an increase of 100%.

EDTA inhibits water moccasin venom pro-

TABLE II. Effects of Various Inorganic Compounds on Digestion of Hemoglobin* by Pit Viper Venom.

Venom		Tyrosine liberated‡			
Water moccasin, mg†	Rattlesnake, mg†	$NaHCO_3$§	$NaHPO_4$§	$Na_2B_4O_7$§ .10 H_2O	NaOH‖
1		6.4	3.9	4.0	3.8
1 + EDTA¶ (pH 7.4 with NaOH)		2.4	1.6	1.6	1.6
1 + EDTA¶ (pH 7.4 with $NaHCO_3$)		2.2			
	2	2.0	.9	.95	1.0

* 5 cc of .8 g % in physiological saline. † Quantity contained in 1 cc. ‡ Millimoles of tyrosine X 10^{-4}/15 min. at 37°C. § Solution originally .02 N adjusted to pH 7.4 with HCl. ‖ Added to all solutions to bring pH to 7.4. ¶ .9 ml of 10^{-3} M conc.

teolysis of urea denatured hemoglobin as it did the liquefaction of gelatin. Sodium bicarbonate in presence of this compound increases tyrosine liberated from 1.6 to 2.4 mM X 10^{-4}, which is an increase of 50%. EDTA brought to pH 7.4 with sodium bicarbonate is at least as active an inhibitor as when sodium hydroxide is used to adjust the pH. Activity of either rattlesnake or water moccasin venom proteases on denatured hemoglobin is approximately the same in presence of either sodium borate, sodium phosphate, or sodium hydroxide when the pH of these solutions is adjusted to 7.4. Although penicillamine and EDTA effectively inhibit the proteolytic action of these venoms, they have no significant effect on toxicity of these venoms in mice.

Discussion. Results reported herewith indicate strongly that activation of venom proteases and reversal of chelating effects by mammalian serum and its ash are due largely to their content of bicarbonate ions. Salts of calcium, magnesium, manganese, cobalt, potassium, ammonium, lithium, iron and copper fail to bring about activation of rattlesnake venom in the absence of bicarbonate ion. Deutsch and Diniz(3), who previously described the action of EDTA on venom proteases, report that inhibition is not reversed by the chlorides of strontium, cobalt, barium, manganese, cadmium, magnesium, zinc, and calcium and nitrates of copper and cobalt in a concentration of 1 X 10^{-2} M.

The possibility of a contaminant being responsible for activation has been considered although only analytical reagent grade chemicals were used. Inactivity of acid ash and acidified sodium bicarbonate, however, is strong evidence for participation of the bicarbonate ion. Total ionic strength, pH, and temperature of all reactions have been carefully controlled.

Activity of EDTA and penicillamine still points in the direction of a cation necessary for venom proteolysis. Reversal of this effect by the bicarbonate ion may be due to indirect inhibition of ability of these compounds to chelate a metallic ion. A close correlation between inhibition of venom proteases and venom toxicity in mice by various serum preparations has been reported(1). The reversal effect of sodium bicarbonate may explain why EDTA and penicillamine are effective proteases inhibitors but ineffective in preventing the lethal effects of the venom in mice.

When fully activated, water moccasin venom is only 6 times as effective as rattlesnake venom on urea denatured hemoglobin with urea removed by dialysis. In previously reported work(1,3) using the same substrate in presence of urea, water moccasin venom was 30 times more effective than rattlesnake venom. They may point to a more rapid denaturation of rattlesnake venom than of water moccasin venom by urea.

Summary. 1. The bicarbonate ion increases proteolytic activity of rattlesnake venom on gelatin layer of photographic film and of both rattlesnake and water moccasin venom on urea denatured hemoglobin with urea removed. 2. The bicarbonate ion also interferes with inhibition of these proteases by EDTA and penicillamine. 3. EDTA and penicillamine have no significant effect on mouse toxicity of these venoms possibly because of bicarbonate content of body fluids.

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Prolongation of Lactation in the Rat by Litter Replacement.* (24839)

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In animals such as cow or goat, in which daily milk production has been measured quantitatively, milk yields reach a peak soon after parturition and then gradually decline. Lactation can be extended beyond the usual milking or weaning period, but level of milk production continues to decline. This is apparently due to decrease in secretion of hormones which stimulate lactation, as demonstrated for prolactin in the rabbit(1), and to progressive involution of mammary secretory tissue(2). Selye and McKeown(3) demonstrated that lactation could be prolonged in 8 postpartum mice by providing them with fresh litters at frequent intervals, but noted involutionary changes in mammary glands after about 30 days and inability to nourish the young after 2 months. The rat normally lactates about 3 weeks after parturition since the young are weaned at this time. It was of interest to determine whether lactation could be prolonged in this species by providing them with fresh litters every 10 days, and to observe effects on mammary involution and milk yields as judged by litter weight gain.

Methods. Eight mature female albino Carworth rats and 8 mature female Long-Evans rats were used. Since no significant differences in lactation were observed in these 2 strains, as judged by litter weight gains, they may be considered as a single population in this experiment. Rats were bred, placed in individual cages, and on day of parturition their litters were standardized to 6 pups/dam. The litters were weighed daily and the dams weighed every 10th day. Every 10 days litters were replaced with equal number of 4-day-old foster pups. In a few cases after 50th day of lactation, it was necessary to replace litters between 9th-14th day, instead of 10th day after previous litter exchange, since some of the 6 young died or new litters were not immediately available. Daily weight gains of litters were averaged for each 10-day interval of lactation. Three rats were killed every 10 days starting on 30th day after parturition, to remove mammary glands for gross and histological examination. The 4 remaining rats were killed on 70th day. Litters were withdrawn from each rat 4 hours prior to autopsy to standardize secretory activity in the alveoli, and right inguinal mammary glands were removed.

Results. By 3rd 10-day interval after parturition the average daily milk yield, as judged by litter weight gains, declined to approximately two-thirds of that observed during 1st 10-day period. Average daily weight gain/litter was 9.6 ± 1.3 g during 1st 10 days and declined to 6.3 ± 1.3 g by 3rd 10-day interval. Thereafter no further decline was observed throughout remaining 40-day interval (Fig. 1). The dams gained in body weight during 70 days of experiment, averaging 262 ± 4 g on day of parturition and 311 ± 4.6 g on 70th day of lactation. This suggests that continued milking did not adversely affect the rats and may have stimulated body growth.

Degree of mammary involution observed was relatively slight in most animals during the 70-day period of lactation. Glands of one of the rats killed at end of 30 days lactation, showed severe alveolar degeneration while glands of 4 rats which had lactated for 70

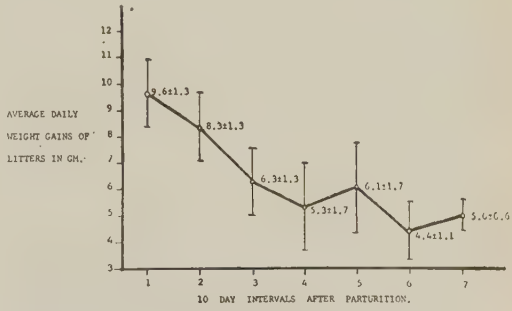

FIG. 1. Avg daily wt gains of litters during successive 10-day intervals. Range represents stand. error.

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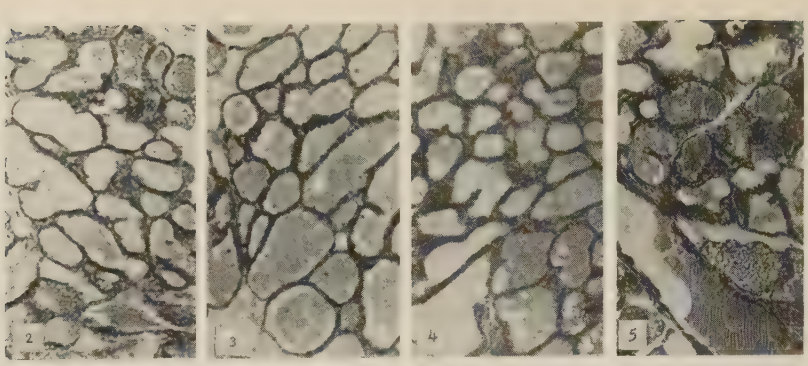

FIG. 2-5. Mammary sections from lactating rats at (2) 4 days, (3) 30 days, (4) 50 days, (5) 70 days. × 63. (Photographs by C. R. Replogle.)

days, showed only slight regressive changes. A few extruded epithelial cells were present in the alveolar lumina of all glands examined. Since most rats showed good preservation of the lobule-alveolar system, mammary involution was probably not the primary factor responsible for decline in milk yield. Figs. 2-5 show histological sections of the mammary glands of rats on days 30, 50, and 70 after parturition, as compared with section taken from rat on 4th day after parturition.

Discussion. Milk secretion was successfully maintained in these rats for 70 days after parturition by regular replacement with fresh litters. Decrease in milk production in these rats may be due to diminished secretion of galactopoietic hormones or a decline in secretory capacity of alveolar cells or both. However, total secretory tissue present during each 10-day period could not be measured and may have been reduced in amount.

Recent investigations by Meites and Nicoll (unpublished)‡ have shown that secretion can be maintained for as long as 75 days after litter removal in postpartum rats, by injecting a combination of prolactin, oxytocin and cortisol. Secretory activity progressively declined, however, and mammary tissue exhibited gradual involution during the 75-day period. Mammary glands of suckled rats in this experiment were much better preserved than in non-suckled, hormone treated rats, probably due mainly to removal of milk, reducing pressure in glands, and possibly also to release of hormones other than prolactin, oxytocin and cortisol by the milking stimulus.

‡ *Fed. Proc.,* 1959, v18, 103.

Bruce(4) recently reported that lactation was prolonged in 3 rats for 9-12 months by frequent litter replacement, and that the litters continued to gain at about the same rate throughout experiment. The latter finding is not in acord with our results, nor with those of Selye and McKeown(3) in mice. It and the present study show, however, that providing rats with fresh litters can greatly extend lactation after parturition. It is believed that litter replacement technic will prove useful for investigations concerned with long term maintenance of lactation and mammary integrity in the rat.

Summary. Lactation was prolonged in 16 rats up to 70 days after parturition by providing them with fresh litters every 10 days. Milk production, as judged by litter weight gains, declined about ⅓ after first 20 days and continued at this level for the remainder of 70-day period. The lobule-alveolar system of most rats showed little or no evidence of involution during entire period. This suggests that the decline in milk yield was due either to decrease in secretion of galactopoietic hormones or to reduction in secretory capacity of alveolar cells, or both.

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Effect of Serotonin upon Liver Cells of Young Rats.* (24840)

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Serotonin (5-hydroxytryptamine) has been studied in connection with many physiologic and disease states, and because patients with carcinoid tumor frequently have acquired valvular lesions of heart(1) there has been great interest in morphologic changes caused by this compound. Prolonged administration of 5-hydroxytryptamine or 5-hydroxytryptophane to experimental animals has resulted in tubular and cortical necrosis of kidneys(2), dermal fibrosis at injection sites(3), gastric mucosal erosions(4), necrosis of tip of tail and of digits, and lenticular opacities(3). There has been no report of morphologic changes in human or animal liver induced by serotonin. With the aid of autoradiographic technic, it has recently been found that in young rats, administration of large doses of serotonin resulted in increased formation of hepatic parenchymal cells, accompanied by minor histological changes. This communication reports these observations.

Materials and methods. Thirty-eight male Sprague Dawley rats‡ weighing 90 to 245 g were maintained on diet of Purina lab chow and water, and housed in individual cages in air-conditioned room. Twenty-five of the animals were given a single subcutaneous injection of serotonin creatinine sulphate§ in 3 ml isotonic saline, the doses ranging 0.009 to 62.8 mg/kg body weight (Table I). Twenty-four hours later, 1 millicurie tritiated thymidine‖/kg was administered by single intraperitoneal injection(5), and after another 4 hours, the rats were killed by decapitation. Thirteen control rats, all litter mates of experimental animals (Table I), were treated in the same way, except that serotonin was replaced by injection of saline. Serotonin and saline injections were performed at same times. The organs were sliced and fixed in cold 10% neutral buffered formalin or in Zenker's solution. After fixing for 24 hours, tissues were washed in running tap water for another 24 hours and then dehydrated, cleared and imbedded in paraffin. Sections of 6-8 μ thickness were prepared and stained as follows: Feulgen's method for desoxyribonucleic acid (DNA), methyl green-pyronin for ribonucleic acid (RNA), hematoxylin and eosin, phloxine methylene blue, periodic acid Schiff's reagent (PAS), diazo coupling reaction for alkaline phosphatase, and Oil Red O stain for neutral fat. For autoradiography, fine grain stripping film¶ was applied over unstained sections and over sections stained with hematoxylin or by Feulgen's method(5). The preparations were exposed in a light-tight box at 10°C for 3 to 4 weeks. Slides were developed in Kodak D-19 Developer 7 minutes, fixed in Kodak Acid Fixer 10 minutes, washed in cool running tap water one hour, and mounted in polyvinylpyrrolidone (PVP).** For histochemical studies, slices of liver were frozen and dried at time of autopsy, using Freed Tissue Dryer, and frozen sections were also cut from formalin fixed tissues. Estimation of mitotic activity and number of hepatic cell nuclei bearing the label was carried out as follows: for each liver the total number of hepatic cells present in 400 X field was determined in sections stained with hematoxylin and eosin. Fifty consecutive 400 X fields were then searched for mitotic figures and results expressed/100,000 hepatic cells. In autoradio-

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† Advanced Research Fellow, Am. Heart Assn.

‡ Purchased from Charles River Breeding Labs., Cambridge, Mass.

§ Supplied by Dr. George Berryman, Abbott Labs. Dosage is expressed in terms of serotonin creatinine sulphate compound.

‖ Schwarz Laboratories, Mount Vernon, N. Y. Specific activity, 0.360 curie/millimole.

¶ Kodak Ltd., London; film AR.10.

** Antara Chemical Co., N. Y.

TABLE I. Autoradiographic and Mitotic Observations in Livers of Rats Weighing Less Than 115 g and Given Single Subcutaneous Injection of Serotonin Creatinine Sulphate (SCS).

Wt, g	Age, days	Dose SCS, mg/kg	Labelled hepatic nuclei*	Hepatic mitoses†
7 control rats given saline (avg of determinations)				
102	36	0	565.7	16.6
10 rats given serotonin				
111	36	.009	581	10
110	36	.09	663	7
111	36	.09	153	4
100	36	1.0	617	5
100	38	2.0	473	27
		Avg	497.4	10.6
107	38	45.0	1,057	42
106	38	47.3	1,090	132
101	38	48.0	1,740	96
97	38	49.5	3,065	495
90	31	62.8	648	72
		Avg	1,520.0	167.4

* No. of tritium labelled hepatic cell nuclei in autoradiographs, expressed/100,000 hepatic cell nuclei.

† No. of hepatic cells in mitosis in hematoxylin and eosin sections, expressed/100,000 hepatic cell nuclei.

graphs, number of labelled liver cell nuclei present in 100 consecutive 400 X fields was counted, and results expressed/100,000 hepatic nuclei. In 7 additional male Sprague-Dawley rats weighing 73 to 98 g, the central lobe of liver was removed under ether anesthesia. Forty-one hours later 1 millicurie of P^{32}/kg body weight was injected by intraperitoneal route. Partial hepatectomy was performed to assure adequate incorporation of P^{32} into nucleic acids of the liver(6,7). Thirteen days later 4 rats were given a single intraperitoneal injection of 40 mg/kg serotonin creatinine sulphate, while the 3 other animals received an injection of saline alone. All 7 rats were killed 24 hours later and the liver homogenized(8). Nucleic acid phosphorus was estimated by method of Schneider(9) and radioactivity of a dried aliquot was determined in a window G.M. counter. Specific activity of nucleic acid phosphorus was expressed as "biological concentration coefficient," *i.e.*, counts/minute/millimole of phosphorus as percent of counts/minute injected/gram of body weight(10). All determinations were performed in duplicate.

Results. The results obtained differed depending on weight of animals. Five rats weighing less than 115 g and treated with 40 mg/kg or more of serotonin creatinine sulphate exhibited increase in number of both hepatic cell nuclei bearing the label and mitotic figures as compared with control rats given only saline (Table I, Figs. 1-3). In a similar group of 5 rats that received smaller doses of serotonin (0.009-2.0 mg/kg), no such difference was observed (Table I). In 15 rats weighing more than 115 g, a statistically significant increase in number of labelled cell nuclei and of mitotic figures could not be demonstrated, even though doses of serotonin exceeded 40 mg/kg.

The increase in labelling appeared to affect only hepatic parenchymal cells; nuclei of bile duct epithelium, of endothelial cells and of Kuppfer cells were labelled to the same extent in livers of treated and control animals. In rats receiving large doses of serotonin (Table I), histologic sections stained with hematoxylin and eosin or with phloxine methylene blue exhibited intact liver cells, but slight disorganization of architecture and slight clumping of cytoplasm was demonstrable. In no instance, however, was frank hepatic cell necrosis observed. In sections stained with Feulgen, PAS, alkaline phosphatase and fat stains, no histological differences were seen between serotonin treated and control animals.

In addition to the liver, the following organs were studied by autoradiography: brain, esophagus, stomach, small and large intestine, skin, heart, lung, spleen, tongue, striated muscle, testis, epididymis, thymus, adrenal, eye, and kidney. In these organs, no significant difference in number of labelled cell nuclei was observed between serotonin treated and control animals, except in the kidney. In rats of all weights given large doses of serotonin, this organ showed an increase in number of labelled nuclei of tubular cells associated with histologic evidence of tubular necrosis.

In 4 rats given P^{32} and serotonin, the "biological concentration coefficient" for nucleic acid phosphorus in liver was 171 (Range 148-189) whereas in 3 control rats that received only saline, the value was 218 (Range 200-237). This difference was significant.

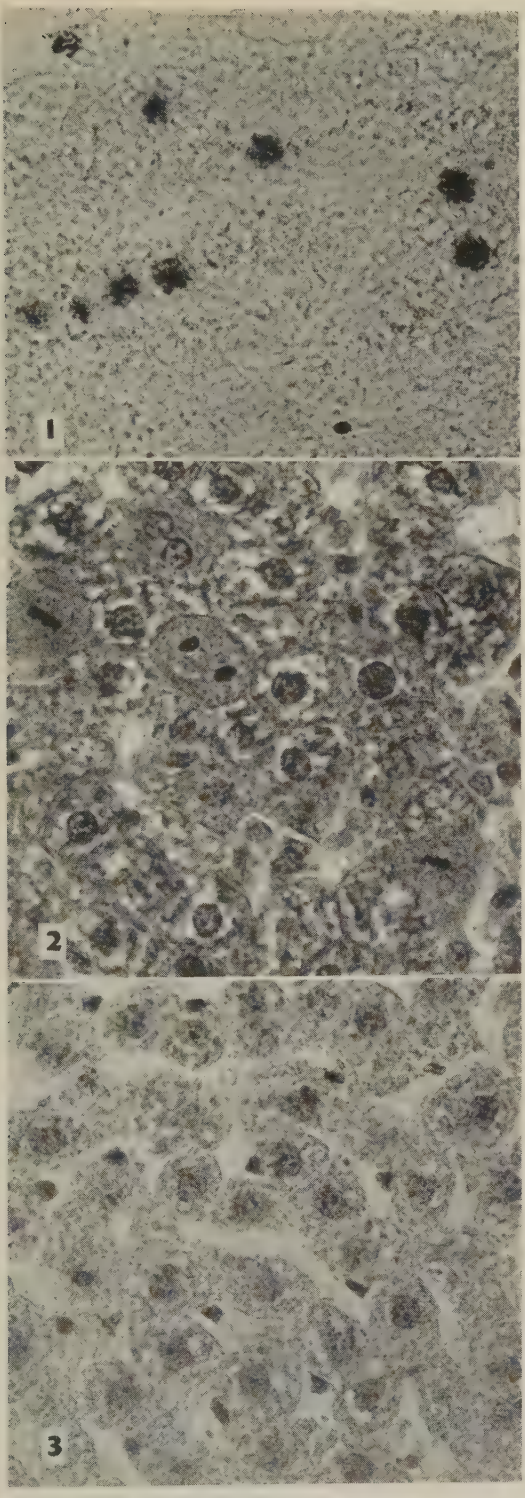

FIG. 1. Autoradiograph of liver of young rat given one subcut. inj. of 45 mg/kg serotonin creatinine sulphate 24 hr preceding inj. of tritiated thymidine. Unstained × 560.

FIG. 2. Liver of same animal as in Fig. 1, showing 3 mitotic figures, slight distortion of cords of liver cells, and clumping of cytoplasm. Hematoxylin and eosin × 560.

FIG. 3. Liver of control rat for comparison with Fig. 2. Hematoxylin and eosin × 490.

Comment. Incorporation of tritiated thymidine into cell nuclei is believed to reflect active nuclear synthesis of desoxyribonucleic acid(5). Thus, it appears that cells which exhibited label in the nucleus were preparing for mitotic division at time of thymidine injection(5). That livers of small rats, given 40 mg/kg or more of serotonin, had increased number of labelled parenchymal liver cells suggests that new liver cells were being formed at increased rate in response to serotonin injection. This is supported by histological observation that mitotic figures were more numerous in these livers than in those of control animals. Moreover, the decreased "biological concentration coefficient" for nucleic acid phosphorus in livers of rats treated with serotonin, suggests an increased turnover of nucleic acid, consistent with above autoradiographic and histologic observations.

The manner in which serotonin affects liver cell proliferation is not understood although the demonstrated dose dependency suggests a toxic effect, acting either directly upon hepatic cells or resulting from temporary ischemia. Furthermore, it is not known why this phenomenon could be demonstrated only in small rats. It is conceivable that serotonin may act by accentuating normal liver growth, which might explain why no significant effect was observed in larger animals.

Summary. In young rats weighing less than 115 g a single subcutaneous injection of 40 mg/kg or more of serotonin creatinine sulphate resulted in increased formation of hepatic parenchymal cells as demonstrated by autoradiographic technics employing tritiated thymidine. With histologic methods, livers showed an increase in mitotic figures. Slight cytologic changes in hepatic cells were observed, but in no instance was frank cellular necrosis found, even with extremely large doses of serotonin. The mechanism by which serotonin affects hepatic cell formation is not

TABLE I. Demonstration of Leukocyte Agglutinating Factor in Serum from Immune Animals.

Avg difference of agglutination index from saline control ± S.E.																			
Immune serum / " cells — Egg albumin, μg/ml			SIII, μg/ml	Nonimmune serum / " cells — Egg albumin, μg/ml			SIII, μg/ml	Immune serum / Nonimmune cells — Egg albumin, μg/ml			SIII, μg/ml	Nonimmune serum / Immune cells — Egg albumin, μg/ml			SIII, μg/ml		
.01	.1	1.0	.01-.1	.1	1.0	10	.01-.1	.5-1.0	.01	.1	1.0	.01-.1	.1	1.0	10	.01-.1	.5-1.0
1.8 ± 1	5.5 ± 1.5	7.8 ± 1	5.0 ± .6	.1 ± .8	1.2 ± .8	4.0 ± .9	.2 ± .8	5.9 ± 1	.9 ± .8	4.1 ± .8	7.2 ± 1.1	4.6 ± .6	.0	.0	5.5 ± 1.1	1.5 ± .9	4.2 ± .6
P = .1	P < .01	P < .01	P < .01	P = .9	P = .15	P < .01	P = .8	P < .01	P = .2	P < .01	P < .01	P < .01			P < .01	P = .15	P < .01

nificant differences represent 58-90% increases over the saline control.

Table II shows that immune serum, when added to normal blood, caused an increased leukocyte agglutination in the presence of specific antigen. Agglutination indices for blood samples containing saline are compared with samples containing immune serum. The highest effective serum dilution is given. Table II also shows that heating such sera before they were added to normal blood destroyed their leukocyte agglutinating activity.

Tables III and IV show adsorption of humoral antibody by specific antigen. Table III indicates EA tanned red cells agglutinated in the presence of anti-EA sera, whereas, saline, bovine serum albumin and bovine serum gamma globulin treated tanned cells did not. When such sera were removed from agglutinated red cells, added to normal blood and their leukocyte agglutinating activities determined in presence of specific antigen, the leukocyte agglutinating activities of sera from only EA tanned cells were diminished (Table III). Table IV shows composite results of 10 experiments in which sera from animals receiving type III pneumococci were adsorbed with both type I and III organisms and their leukocyte agglutinating activities tested. It is apparent that only type III organisms diminished the leukocyte agglutinating activity of such sera.

Discussion. Previous work has shown that, although LAA can be demonstrated in blood of animals susceptible to anaphylactic shock, the anaphylactic type of hypersensitivity is not a prerequisite for its presence(5). Likewise, LAA can be demonstrated in serum of animals without detectable precipitating antibody(5). This suggests that either the LAA and precipitating antibody are different or the technic for detecting classical antibody by leukocyte agglutination is relatively more sensitive than usual methods for detecting precipitating antibody. The work here presented has not attempted to answer this question, but to determine whether the LAA is humoral or cellular and whether it behaves as an antibody. That LAA is humoral has been shown by 3 types of experiment. Cell-serum cross ex-

TABLE II. Titers of Immune Sera Producing Leukocyte Agglutination in Normal Blood with Specific Antigen. Effect of heat on leukocyte agglutinating activity of such sera.

	Agglutination index				Agglutination index		
Exp.	Saline	Serum	Serum titer	Exp.	Saline	Untreated serum	56°C serum
EA 1	57	75	1/500	EA 7	16	24	11
2	28	48	"	8	17	23	20
3	53	66	1/1000	9	31	52	28
4	21	31	1/500	10	24	33	23
5	14	40	1/1000	11	21	38	20
6	41	54	"				
SIII 1	8	15	"	SIII 9	15	24	17
2	9	17	1/500	10	31	46	35
3	12	18	1/1000	11	43	69	40
4	1	6	"	12	17	28	19
5	1	9	"	13	28	45	23
6	1	7	1/5000	14	27	42	27
7	4	13	1/500				
8	4	11	"				

Final antigen concentration: EA, 1 μg/ml; SIII, .1 μg/ml.

periments have shown that nonimmune cells + immune serum react at antigen concentrations which characterize an immune system, whereas, immune cells + nonimmune serum react at antigen concentrations characterizing a nonimmune system. LAA can be transferred *in vitro* to normal blood by adding small amounts of immune serum and such activity can be destroyed or impaired by heating such sera at 56°C. Finally, LAA can be specifically adsorbed from immune serum. It must be concluded that with the experimental model used there was no evidence for cellular antibody.

There is also convincing evidence that the humoral agent producing leukocyte agglutination behaves as an antibody. Immunization resulted either in formation of a new unit capable of reacting with antigen or increased the number of preexisting units capable of reacting with antigen above a critical threshold, which was adequate to trigger leukocyte agglutination in the immune system. Also, from Tables III and IV, the humoral factor is shown to have an affinity for specific antigen. The factor occurring in anti-EA sera was adsorbed by EA tanned red cells but not by tanned cells treated with saline, bovine serum albumin or bovine gamma globulin. Similarly, the factor occurring in anti-type III

TABLE III. Effect of Adsorption by Specific Antigen on Leukocyte Agglutinating Activity of Immune Serum.

	Reciprocal hemagglutination test titers				Leukocyte agglutination index (EA, 1 μg/ml)					
Serum	EATC	STC	BGGTC	BSATC	Saline	Non-adsorbed serum	STC serum	EATC serum	BGGTC serum	BSATC serum
EA 1	4096	Neg.			31	65	60	44		
4	1024	"			9	29	26	8		
7	4096	"			26	45	39	24		
8	4096	"			15	lost	27	11		
9	8192	"			8	19	16	6		
11	16,384	"	Neg.	Neg.	20	28	25	21	27	26
12	2048	"			27	31	34	20		
13	16,384	"	"	"	6	11	12	6	12	13
16	128	"			38	106	102	74		
17	4096	"			50	60	65	47		
Normal	Neg.	"								
SIII	"	"								

(EATC) egg albumin tanned cells; (STC) saline tanned cells; (BGGTC) bovine gamma globulin tanned cells; (BSATC) bovine serum albumin tanned cells.

TABLE IV. Difference of Average Agglutination Index Due to Adsorption of SIII Immune Serum by Type I and Type III Pneumococci.

Significance between	Difference ± S.E.	P
Saline and nonadsorbed serum	6.2 ± .8	<.01
Saline and Type III adsorbed serum	1.1 ± 1.0	.30
Nonadsorbed serum and Type III adsorbed serum	7.3 ± 1.2	<.01
Saline and Type I adsorbed serum	8.4 ± 1.2	"
Type I adsorbed serum and Type III adsorbed serum	9.5 ± 1.4	"

SIII, .1 μg/ml.

pneumococcal sera was adsorbed by type III pneumococci but not by type I. From the work of Borduas and Grabar(9) it may be questioned whether the EA tanned cells really adsorbed anti-EA antibody, since these workers claim that tanned cells preferentially adsorb conalbumin rather than EA. Stravitsky has shown that EA is adsorbed about as well as conalbumin(10). The fact that this issue may be controversial does not invalidate the results of our study. Whether EA or one of its impurities was the principal antigenic unit, LAA was formed and adsorbed from serum by antigenic unit fixed to tanned red cells.

Waksman reported that rabbit anti-ovalbumin sera produced a decrease in leukocyte count when added to normal blood in presence of specific antigen(11). He found no correlation between white cell lytic activity of such antisera and precipitating antibody but did find some correlation with skin sensitizing antibody. It is likely that the antibody involved in decreased leukocyte count observed by Waksman and the LAA are identical. However, the nature of LAA and its exact relation to antibody of known type remain to be elucidated.

Summary. A humoral agent produced by single injection of antigen and responsible for leukocyte agglutination, which occurs in presence of specific antigen has been demonstrated in anti-EA and anti-type III pneumococcal sera. The factor is heat labile and, due to its specific affinity for EA treated tanned red cells and type III pneumococci respectively, it is thought to be antibody.

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Histochemical Demonstration of Erythrocyte Esterases.* (24842)

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Although the histochemical demonstration of esterases in tissues can be performed by means of azo and indoxyl technics, such technics have not been applied successfully to erythrocyte esterases(1). The demonstration of acetylcholinesterase, based on the Koelle method, was noted by Zajicek *et al.*(2); the precipitate, however, is crystalline and localization of enzyme activity within the erythrocyte has not yet been reported. During preliminary studies on development of high resolution histochemical technics for intracellular localization of enzymes, erythrocyte esterase activity could be demonstrated with excellent localization in ordinary air-dried blood smears.

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The salient characteristics of the technic are, (1) low temperature anhydrous osmium tetroxide fixation and, (2) use of "hexaazotized" pararosanilin in an azo coupling reaction(3,4).

Method. In our procedure, blood smears are first fixed in anhydrous osmium tetroxide, then incubated in a medium containing alpha naphthyl acetate and "hexaazotized" pararosanilin. Esterase enzymes present in the erythrocyte split the alpha naphthyl acetate to alpha naphthol and acetate ion. The "hexaazotized" pararosanilin then couples with alpha naphthol to produce a colored deposit at sites of enzyme activity. Discrete intracellular erythrocyte esterase activity has been demonstrated in blood smears from garter snake, frog, mouse, rat, rabbit and man.

Materials. (1) Fixative. 1% osmium tetroxide in dimethylformamide (DMF)†. DMF available as reagent grade chemical contains trace quantities of substances which reduce enzyme activity and which react with osmium tetroxide. Purification and dehydration are readily achieved by passage of DMF through freshly activated charcoal and then through Linde Molecular Sieve Powder, Type 4A.‡ The treated DMF is colorless and has a faint sweet odor. 1% solution of osmium tetroxide in DMF is prepared (in a hood). The deep yellow solution is stored in screw cap Coplin jar in freezing compartment of refrigerator at temperature of about –15°C. The solution is stable indefinitely at this temperature. Repeated use eventually introduces contaminants which react with osmium tetroxide as indicated by gradual appearance of a brown-black color. (2) Buffer solutions. 1/15 M Na_2HPO_4, 1/15 M KH_2PO_4. (3) Substrate. Alpha naphthyl acetate, reagent grade.§ (4) The "hexaazonium" salt is made prior to incubation by mixing hydrochloric acid solution of pararosanilin with sodium nitrite solution. (A) One gram of pararosanilin hydrochloride (C.I. 676) is added to 25 ml of 2N HCl, warmed to dissolve the dye, filtered and stored in dropper bottle at room temperature. (B) One gram of sodium nitrite is dissolved in 25 ml of distilled water and stored in dropper bottle at room temperature. (5) Slides. Thin blood smears are made. If smears are not used the same day they should be stored in refrigerator. Activity can be preserved for several months at 5°C. *Procedure.* (1) Fixation. Dry blood smears are placed in Coplin jar containing osmium tetroxide in DMF for 2 to 3 minutes at –15°C. At completion of fixation the slides are removed one at a time and *immediately* placed in stream of cold, briskly running tap water for 15 seconds to remove fixative. (If a refrigerated slide is to be fixed, care must be taken to prevent water vapor condensation on the slide since the introduction of a moist smear into the fixative produces, in effect, aqueous osmic acid fixation which inactivates erythrocyte esterases. Care also must be taken when the slide is removed from the fixative; unless the fixative is washed off without delay, water vapor condensation on the slide in the presence of osmium tetroxide causes rapid inactivation of the enzymes.) (2) Incubation medium. 25 ml of 1/15 M Na_2HPO_4 are added to Coplin jar. About 15 mg of alpha naphthyl acetate are placed in test tube, heated under hot water tap until the ester begins to melt (about 45°C). 2 to 3 ml of hot water are added to tube and the tube shaken. The resultant turbid mixture is transferred to the Coplin jar with stirring to give a saturated solution. "Hexaazonium" salt is made by adding 4 drops of sodium nitrite solution to 4 drops of pararosanilin-hydrochloric acid solution in a separate test tube and the tube agitated. "Hexaazotization" is complete in about 30 seconds and the amber solution is transferred to Coplin jar with stirring. pH of medium is now adjusted by pH meter to 7.3 by addition of 1/15 M KH_2PO_4.‖ (3)

† Osmium tetroxide purchased from Engelhard Industries, Newark, N. Y., Dimethylformamide purchased from Matheson Coleman and Bell, East Rutherford, N. J. and from Distillation Products, Rochester, N. Y.

‡ Linde Molecular Sieve Powder, Type 4A purchased from Linde Air Products, N. Y.

§ Alpha naphthyl acetate purchased from Distillation Products, Rochester, N. Y.

‖ Selection of pH 7.3 was based on qualitative observations and represents a necessary but satisfactory compromise. It was found that, within limits, as pH of incubating medium was raised, erythrocyte esterase activity increased but rate of enzyme inacti-

Incubation. The slides (up to 5) are placed in Coplin jar and incubation performed at room temperature (approximately 25°C). Incubation time varies with species of organism. Erythrocytes of garter snake are incubated for about 5 minutes; rabbit for about 10 minutes; frog, mouse, rat and man for about 60 to 120 minutes.‖ At end of incubation the slides are washed in water, passed through acetone to remove naphthyl acetate which may have deposited on the preparation, and allowed to dry.

Results. Sites of erythrocyte esterase activity are demonstrated by presence of azo dye formed by coupling of "hexaazotized" pararosanilin and alpha naphthol, the latter component formed by enzymatic hydrolysis of alpha naphthyl acetate.¶ The final dye product is red-brown and insoluble in water, acetone, alcohol, benzene and all other solvents tested. It is stable and it does not diffuse in permanent mounting media. The dye is amorphous and clearly delineated against the background of erythrocyte cytoplasm.

No dye deposit is found if enzymes are inactivated, *e.g.,* by flaming the smear, or if either substrate or "hexaazonium" salt is omitted from incubating medium.

Non-specific coupling of "hexaazonium" salt to proteins imparts a pale yellow color to proteins previously colorless and a light orange color to erythrocyte hemoglobin.

Differences in morphologic distribution of dye deposits within erythrocytes and in number of erythrocytes showing activity are readily observed. In general the morphologic distribution of enzyme activity is of 2 types: (1) [Fig. 1A] spherical granules which may vary both in number (from 1 to 20) and in size in a particular erythrocyte and (2) [Fig. 1B] an irregular mass with filamentous exten-

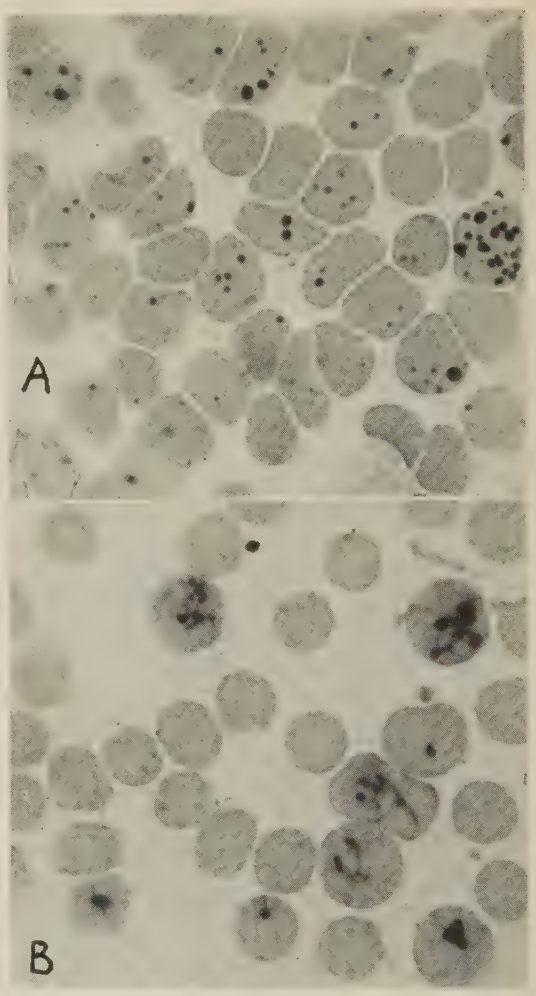

FIG. 1. Erythrocyte esterase in peripheral blood smears (×1134). A. Human with polycythemia vera. B. Rabbit during phenylhydrazine induced anemia.

vation by 'hexaazonium' salt, as with all azo couplers, also increased. At pH 7.3 at room temperature dye deposition from enzymatic hydrolysis proceeds at "adequate" rate for about 4 hours. At pH 6.9 rate of enzymatic hydrolysis is slower; however, 'hexaazonium' inactivation of the enzyme is further depressed: the net effect is an increase in detection sensitivity provided incubation is extended to 6 to 16 hours. Selection of pH and incubation time is, therefore, arbitrary and depends upon type of information required. Incubation at pH 7.3 has been satisfactory for all species of erythrocytes studied for unequivocal demonstration of positivity and for detection of differences between normal controls and abnormal specimens. If, on the other hand, greater sensitivity in enzyme detection is required it is necessary to perform the incubation at pH 6.9.

¶ Leucocytes and platelets are demonstrated by this technic. Optimum pH of incubating medium for leucocytes is 6.9. Human lymphocytes, when positive, contain a few distinct granules in the cytoplasm; monocytes, a fine diffusely distributed stippling of cytoplasm; neutrophils, a faint, diffuse activity throughout cytoplasm after at least 4 hours incubation. Of interest is the finding that rabbit pseudoeosinophil nuclei are positive.

sions, often forming an elaborate network. Spherical granules are often found attached to the filamentous extensions. Both types may be seen in the same blood smear.

Work in progress indicates that induced or naturally occurring diseases involving erythrocyte production are often associated with variations in enzyme activity. Thus, preliminary studies show an appreciable increase in number of cells containing both types of dye distribution in rabbits during the phase of reticulocytosis following phenylhydrazine induced anemia [Fig. 1B]. In humans with polycythemia vera the number of erythrocytes containing spherical granules and number of granules/cell are strikingly increased over normal controls [Fig. 1A]. Similar findings have been noted in some types of anemia in humans.

Discussion. Previous failures to demonstrate erythrocyte esterases by histochemical azo technics may have been due to inadequate fixation and/or inadequate detecting systems.

Anhydrous osmium tetroxide is an excellent fixative for blood smears(3). When used at low temperatures sufficient enzyme activity is preserved to keep incubation time within a reasonable range. Relative stability of "hexaazotized" pararosanilin and of alpha naphthyl acetate against spontaneous hydrolysis contribute to sensitivity of the system by reducing artifactual colored precipitates to a minimum. Rapidity of the coupling reaction, (and extreme insolubility) and high extinction coefficient of the coupling product result in precise localization and high sensitivity. Demonstration of enzyme activity in filaments so fine as to approach or possibly exceed the limit of resolution of the light microscope and absence of dye diffusion halos or crystalline deposits are evidence of high resolution of the technic.

Erythrocytes are known to contain at least 3 different types of esterases: acetylcholinesterase, cholinesterase and ali-esterase. Employing acetylcholine as substrate in biochemical assays, other workers have noted a correlation between erythrocyte esterase and reticulocytosis in rat and man(5,6). A similar correlation has been found here in rabbits by means of histochemical azo technic.

Histochemical observation of increased esterase activity in polycythemia vera erythrocytes requires confirmation by conventional quantitative methods.

Since the substrate, alpha naphthyl acetate, can be split by all 3 erythrocyte esterases(7) and since anhydrous osmium tetroxide may differentially preserve activity of different enzymes, further work is necessary to identify specific enzymes responsible for dye deposits seen in this histochemical technic.

Summary. A high resolution technic is described for localization of intracellular sites of erythrocyte esterases. Blood smears are fixed in 1% osmium tetroxide in dimethylformamide at −15°C and then incubated in medium containing alpha naphthyl acetate and "hexaazotized' pararosanilin. Alpha naphthol released by enzymatic hydrolysis of the substrate ester couples with "hexaazonium" salt to form a colored, insoluble, amorphous deposit at sites of esterase activity.

ADDENDUM. During the writing of this paper an article appeared in *J. Histochem. and Cytochem.*, 1958, v6, 457, by M. Wachstein and G. Wolf entitled: Histochemical Demonstration of Esterase Activity in Human Blood and Bone Marrow Smears.

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Homologous Skin Transplantation from F_1 Hybrid Mice to Parent Strains.* (24843)

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Transplantation of normal and neoplastic tissues in highly inbred strains of mice is ruled by basic principles of inheritance. Tumors as well as normal tissues taken from animals of an inbred strain can be successfully transplanted into the same individual (autologous graft) or into members of the same strain (isologous grafts). In addition, by crossing mice of 2 different strains, the resulting F_1 hybrids accept transplants of tissue taken from members of either parental strain. Conversely, tissues taken from F_1 individuals and transplanted to members of either parental strain are always unsuccessful and result in a homograft rejection(1,2,3). However, incidental observations made recently in our laboratories seem to indicate that using certain "F_1 to parent" donor host combinations and under certain well defined conditions, such as transplantation in young recipient animals, skin from F_1 hybrid individuals can be successfully transplanted onto members of at least one of the parental strains. It is the purpose of this report to document these observations.

Method. Highly inbred mice of the Z,† A, BALB/C, Ce, C57 B1 (Subline 1) and their reciprocal hybrids were used. In a first set of experiments, skin taken from F_1 hybrid mice was transplanted to groups of mice of each parental strain, as follows: (A x Z) F_1 onto A and Z mice; (BALB/C x Ce) F_1 onto both BALB/C and Ce: (Z x C57 B1) F_1 onto Z and C57 B1 and (Z x Ce) F_1 or (Ce x Z) F_1 onto Z and Ce parental strains. Donors as well as recipient animals were divided into subgroups according to age at time of skin graft. These ages are indicated in Table I. In these experiments, donors of grafts and recipients were always of same sex. Method used for skin transplantation has been described(4). Essentially, it consisted of transfer of full-thickness abdominal skin graft from donor to back of recipient. In each instance the graft was turned 180° to facilitate determination of subsequent success or failure of graft. In such grafts hair on successful graft grows in a direction opposite to that on skin of host. Grafted animals were kept under observation for at least 5 months following operation. Mice were housed individually in plastic boxes and fed Purina Fox Chow and tap water.

Results. The results are recorded in Table I. When skin from (A x Z) F_1 hybrids was transplanted to 26-38 days old mice of the Z strain, and to A mice 25 days of age, none accepted the hybrid skin. The same was true when (BALB/C x Ce) F_1 skin was transplanted to mice of BALB/C or Ce strains and when (Z x C57 B1) F_1 skin was grafted onto mice of either C57 B1 or Z parental strains.

Similar negative results were obtained when (Z x Ce) F_1 skin was grafted onto Ce mice. However, when skin from this hybrid was grafted onto Z mice of 26 to 47 days of age, 28 of 34 animals (82%) accepted the graft which remained in place at least 5 months. Of this group, 9 animals were then retransplanted with skin from the same F_1 hybrids, when they reached approximately 5 months of age. This was done by replacing previous graft with another piece of hybrid skin. All of these recipient mice also accepted the second skin graft. On the other hand, when the recipient Z mice were 55 to 61 days of age at time of first grafting, only 12 of 31 (39%) accepted the graft. In contradistinction, none of 19 animals of the same parent strain (Z), ranging from 83 to 119 days in age, accepted this (Z x Ce) F_1 skin. It is interesting to note that the reciprocal hybrid skin resulting from the cross between Ce mothers and Z fathers, transplanted onto young Z mice, was also successful in 8 of 11 mice tested.

* Aided by grants from U.S.P.H.S. and Minn. Division, Am. Cancer Soc.

† "Z" is used to simplify designation of mice of the C3H strain originally obtained from Dr. John J. Bittner's Laboratory.

TABLE I. Incidence of Successful Skin Grafts from F_1 Hybrid Mice to Individuals of Either Parent Strain.

Donor hybrid	Age in days	Recipient parent strain	Age in days	No. of mice accepting skin grafts*	No. of mice accepting 2nd skin graft*
(A × Z)F_1	37–100	Z	26– 38	0/12	
		A	25	"	
(BALB/C × Ce)F_1	37– 42	BALB/C	27– 67	0/14	
		Ce	26	0/10	
(Z × C57 Bl)F_1	33– 90	C57 Bl	28– 38	0/12	
		Z	25– 43	0/19	
(Z × Ce)F_1	29– 95	Ce	33– 56	0/25	
		Z	26– 47	28/34	9/9
		Z	55– 61	12/31	
		Z	83–119	0/19	
(Ce × Z)F_1	28	Z	32	8/11	

* No. +/No. grafted.

In view of these results, a second series of experiments was performed consisting of exchange of skin grafts between members of both Z and Ce parent strains.

The results of this experiment are recorded in Table II. It is clear that when Ce mice were used as donors and Z mice as recipients, rejection of the homograft regularly occurred regardless of age of recipient. Likewise, Ce mice always rejected Z skin.

Induction of acquired tolerance to (Z x Ce) F_1 skin homograft in weanling Z mice. Heretofore, all instances of immunological tolerance have been induced by injection of cells from prospective donor to prospective recipient prior to birth or in the immediate neonatal period(5-10). Since most Z mice ranging from 26 to 47 days of age accepted skin transplants from (Z x Ce) F_1 hybrids, whereas older animals, particularly those in 83 to 119 days age group always rejected this skin (Table I), it was decided to determine whether or not intravenous administration of viable spleen cells, taken from (Z x Ce) F_1 donors and injected intravenously into weanling mice of the Z strain, would induce permanent state of tolerance to the F_1 hybrid.

TABLE II. Homologous Skin Grafts between Mice of Z and Ce Strains.

Donor strain	Age in days	Recipient strain	Age in days	No. of mice accepting skin grafts*
Ce	60-180	Z	23-80	0/29
Z	44- 80	Ce	31-80	0/23

* No. +/No. grafted.

The experiment was performed in the following way: Weanling female and male mice of the Z strain from 21 to 25 days of age were injected intravenously into tail vein with 15-20 million viable spleen cells taken from (Z x Ce) F_1 adult donors. Donors were killed under ether, the spleen removed and cells to be injected prepared according to technic previously described(4). Spleen cells for each injection were suspended in 0.25 cc of Ringer-Locke's saline solution.

TABLE III. Induction of Tolerance to (Z × Ce)F_1 Skin Homograft in Weanling Z Mice Injected Intravenously with (Z × Ce)F_1 Spleen Cells.*

Host strain	Age (days) when inj. with F_1 spleen cells	Age (days) when grafted with F_1 skin	No. of mice accepting skin grafts†
Z	21-25	100-130	14/15
"		83-119	0/19

* Each mouse received 15 to 20 million viable cells.

† No. +/No. inj.

The injected mice, together with a group of non-injected controls, were set aside until they reached from 100 to 130 days of age, at which time they were grafted with skin from (Z x Ce) F_1 donors of approximately the same age. In all instances recipient and donor mice were of the same sex. After grafting, both experimental and control mice were kept under observation for no less than 5 months.

The results are recorded in Table III. Of 15 Z mice injected immediately after weaning with F_1 spleen cells, 14 or 93% accepted the corresponding F_1 graft. In contradistinction,

all 19 control animals of approximately the same age rejected the skin grafts in the usual way.

Discussion. The results of these experiments confirm that in most instances the skin taken from F_1 hybrid individuals resulting from crossing 2 different strains of mice can not be successfully transplanted onto animals from either parent stock. However, when skin taken from (Z x Ce) F_1 hybrid was grafted onto young Z mice 26 to 47 days of age, a high incidence of successful takes was achieved. On the other hand, when skin from the same donor hybrid was grafted to older recipients of parent strain, the incidence of successful grafts decreased markedly, and in fully mature animals grafts failed altogether. The differences in incidence of successful takes in the 3 groups studied are highly significant on a statistical basis. Similarly, the reciprocal F_1 hybrid skin taken from (Ce x Z) F_1 animals also was accepted in most cases when transplanted onto young mice of the Z strain.

It is further of interest that mice accepting the first homologous skin graft remained susceptible to a second transplant of the same skin performed at an age when animals of the same strain not subjected to a previous graft regularly rejected the homologous skin, suggesting that persistence of initial graft induced in recipients some degree of tolerance to the skin of homologous hybrid strain.

Further evidence for this hypothesis was obtained in the observation that injecting weanling Z mice with viable spleen cells from adult (Z x Ce) F_1 produced immunological tolerance, permitting the parent Z strain recipients to accept skin from hybrid donor at any age.

It is difficult with present concepts to offer a satisfactory explanation of these results. It seems possible, however, to postulate that, of strain combinations studied, Ce and Z mice differ from each other by weak histocompatibility genes(11,12) which are, however, sufficiently strong to permit regular rejection of skin homotransplants between the 2 parent strains. However, when strains are crossed, the histocompatibility differences between hybrid and parent strain may be lessened by genetic mechanisms such as those postulated by Fox(13). This might make it possible for the recipient strain to accept homografts or develop tolerance to the hybrid more easily than would be the case between parent strains. The observation that in these combinations the young recipients of parent strain accept such homografts from donors of the hybrid strain, whereas older recipients do not, would be best explained by the hypothesis that young animals, having not reached complete immunological maturity, are more susceptible to both homotransplantation and to development of immunological tolerance than are older animals of the same strain.

Summary. 1) Using several "F_1 hybrid to parent" strain combinations homotransplantation of skin from F_1 hybrid mice onto members of both parent strains has been attempted. 2) Results showed that while most "F_1 to parent" homologous skin grafts were unsuccessful, skin taken from (Z x Ce) F_1 or (Ce x Z) F_1 grafted onto mice of Z parent strain 26 to 47 days of age, was successfully established in most cases. Moreover, older recipients of same strain, particularly those ranging from 83 to 119 days in age, rejected the F_1 skin in all instances. 3) Exchange of skin homografts between mice of Z and Ce strain was regularly unsuccessful regardless of recipient's age at time of grafting. 4) Acquired immunological tolerance to (Z x Ce) F_1 homologous skin grafts was achieved in weanling Z mice injected intravenously with viable spleen cells taken from adult F_1 donors.

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Reduced Growth Hormone Content in Anterior Pituitaries of Rats on Protein-Free Diets.*† (24844)

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Diets inadequate in protein or lacking specific essential amino acids retard skeletal growth. Several investigators(2-10) have measured tibia length and width of epiphyseal cartilage plates to assess growth retardation during such deficiencies in the rat. Frandsen *et al.*(8) found that in complete absence of dietary protein, the tibial proximal epiphyses were sealed from shaft by bone, known to occur after hypophysectomy(11). To determine whether growth arrest in absence of dietary protein was due to decreased synthesis of growth hormone, anterior pituitaries of protein-deficient rats were bioassayed for growth hormone activity and studied histologically. An attempt was also made to measure growth-promoting potency of blood from such animals to gain additional information on pituitary function.

Methods. Adult female rats (Long-Evans strain) weighing approximately 200 g were maintained 5 weeks on purified diet free of protein, though complete in all other known dietary essentials.‡ The animals had lost an average of 65 g. Groups of control rats of similar age and body weight were maintained either on purified diet containing 24% casein‡ or on stock diet XIV(13) and during same period gained 40-50 g. Bioassay results for control groups were in such close agreement that they have been combined in the Tables. Anterior pituitaries from protein-deficient and control rats were dissected, weighed, ground, suspended in isotonic saline with 2% butanol, and stored at 4°C. Total doses ranging from 1/32 to ½ anterior lobe were injected subcutaneously into immature female hypophysectomized rats. Injections were given once daily for 4 days, followed by autopsy 24 hours after last injection. The right tibia was stained with silver nitrate(14). Width of uncalcified portion of proximal epiphyseal cartilage was measured with ocular micrometer and cartilage widths of 200 μ or more, *i.e.,* increase of at least 40 μ over uninjected control widths, were considered evidence for growth-stimulating activity. Plasma from protein-deficient and control rats was bioassayed by same method(14). Blood was collected for 4 consecutive days from the aorta in heparinized syringes and centrifuged under mineral oil 30 minutes at 3200 rpm. Plasma for each day was pooled and injected subcutaneously

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‡ The protein-free diet contained sucrose 88%, hydrogenated cottonseed oil 8%, modified salts No. 4 (12) 4%. Crystalline vit./kg of diet were: vit. B_{12} 0.05 mg, *d*-biotin 0.6 mg, 2-methyl-1,4-naphthoquinone, 10 mg, thiamine HCl 10 mg, pyridoxine HCl 10 mg, pteroylglutamic acid 11 mg, riboflavin 20 mg, p-aminobenzoic acid 20 mg, niacin 40 mg, *d*-calcium pantothenate 100 mg, inositol 800 mg, choline chloride 1000 mg. The control diet contained sucrose 64%, alcohol-extracted casein 24%, hydrogenated cottonseed oil 8%, modified salts No. 4(12) 4% and vit./kg of diet listed above with B_{12} omitted. All rats received weekly a fat-soluble vit. supplement of 800 U.S.P. units vit. A, 115 chick units vit. D, 6 mg synthetic alpha-tocopherol and 650 mg corn oil (Mazola).

TABLE I. Growth Promoting Activity of Rat Anterior Pituitaries.

Total fraction of gland	Normal donors: Wt of gland, mg	Normal donors: No. of recipients*	Normal donors: Body wt change, g	Normal donors: Tibial cartilage width, μ	Protein-deficient donors: Wt of gland, mg	Protein-deficient donors: No. of recipients	Protein-deficient donors: Body wt change, g	Protein-deficient donors: Tibial cartilage width, μ
0	.0	33	−1	158 ± 4†	.0	29	−1	152 ± 4
1/32	.3	24	1	181 ± 5	.2	10	−1	166 ± 5
1/16	.6	23	3	220 ± 6	.3	10	1	163 ± 4
1/8	1.1	22	5	236 ± 8	.6	31	1	190 ± 6
1/4	2.3	21	6	269 ± 9	1.2	31	3	208 ± 7
1/2	4.5	22	7	283 ± 9	2.4	32	3	243 ± 8

* Immature female rats, hypophysectomized 26-28 days of age, 16-33 days post-operative and maintained on stock diet I plus lettuce(13).

† Stand. error of mean.

into immature hypophysectomized rats in doses of 2 to 8 ml. The larger doses were administered in 2 or 3 ml at 1-2 hour intervals. Autopsy was performed 24 hours after last injection. Groups of 2 to 4 rats were used for each dose level and every level was repeated at least twice. Pituitary glands for histological analysis were collected from additional donor animals and fixed in Zenker-Formol, embedded in nitro-cellulose, sectioned at 4 μ, and stained with Mallory-Azan(15).

Results. Anterior lobes of control rats proved consistently more potent in increasing width of tibial epiphyseal cartilages in recipient animals than glands of protein-deficient rats (Table I). Growth-promoting activity could be detected in 1/32 to 1/16 total dose of anterior lobe from control rats, whereas 1/8 to 1/4 of gland was the minimal effective dose for such activity in pituitaries of protein-deficient rats, *i.e.*, in protein-depleted rats the pituitary contained approximately 1/4 normal amount/gland or 1/2 amount/mg tissue.

Cells of anterior pituitary of protein-deficient rats were reduced in size as judged by increased number of nuclei in any given field (Figs. 1 and 2). There were fewer acidophils in anterior lobes of protein-depleted group than in comparable areas of control pituitaries. The acidophils present were small and stained less intensely, presumably due to degranulation.

Plasma from protein-deficient donors appeared less effective in stimulating epiphyseal cartilages of recipient rats than plasma from control rats (Table II). However, evaluation of data was difficult due to marked variability in response of test animals. Results obtained with higher dosage levels (6 to 8 ml plasma daily) could not always be repeated, perhaps

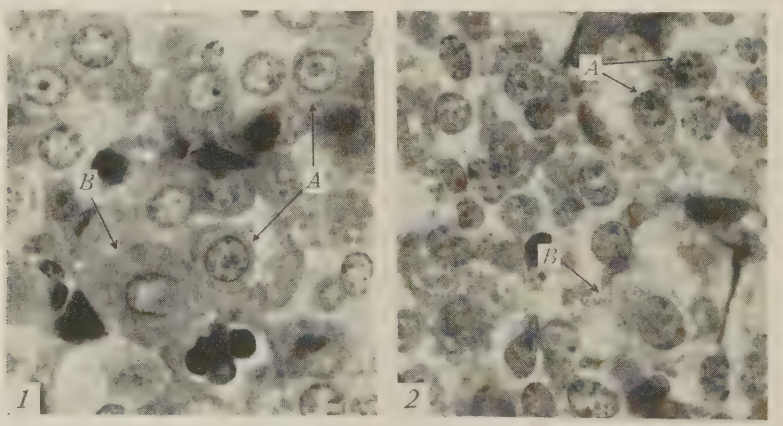

FIG. 1. Photomicrograph of portion of anterior pituitary of female rat on control diet showing general distribution of acidophils (A) and basophils (B). Mallory-Azan (× 890).

FIG. 2. Photomicrograph of portion of anterior pituitary of female rat on protein-free diet. Note increased number of nuclei. Acidophils are reduced in number and size. Mallory-Azan (× 890).

TABLE II. Growth Promoting Activity of Rat Plasma.*

Total dose, ml	Normal donors: No. of recipients	Normal donors: Body wt change, g	Normal donors: Tibial cartilage width, μ	Protein-deficient donors: No. of recipients	Protein-deficient donors: Body wt change, g	Protein-deficient donors: Tibial cartilage width, μ
0	22	−3	162 ± 3	23	1	163 ± 5
8	13	0	182 ± 4			
16	16	2	202 ± 7	9	2	180 ± 8
24	10	2	221 ±10	10	1	206 ± 8
32	6	1	252 ± 9	7	1	210 ± 7

* Abbreviations as in Table I.

due to variable absorption of such volumes. A high mortality rate occurred in groups receiving larger doses, whereas there were no deaths among recipients receiving smaller volumes.

Discussion. Reduced growth hormone content of the hypophysis during protein deprivation may signify decreased synthesis of growth hormone, an assumption supported by reduction in number and size of pituitary acidophils. Similar changes have been described previously in pituitaries of young male rats deprived of certain essential amino acids(6,7, 9,10,16). Decrease in growth-stimulating activity of blood in this study would not be unexpected under those circumstances.

Failure of growth hormone synthesis in absence of dietary protein may not be the only cause of growth retardation. Lack of amino acids as "building blocks" due to protein deprivation is undoubtedly a limiting factor in skeletal growth. Inability of skeleton to respond to growth hormone may also be a factor in growth arrest in protein-deficient rats, for it is known that the response of hypophysectomized rats to injected growth hormone, as judged by gain in body weight, varied with quality and quantity of protein fed(17). Administration of pituitary growth hormone has been reported to stimulate inactive epiphyseal cartilage plates in intact rats fed diets deficient in tryptophane(18) or in phenylalanine and tyrosine(19); no such effect has so far been demonstrated in complete absence of dietary protein.

Summary. Anterior pituitaries of protein-depleted and of control female rats were bioassayed for growth-stimulating activity. Absence of dietary protein for 5 weeks reduced growth hormone content of hypophysis to ¼ that found in normal glands or ½ normal amount/mg tissue. There was a parallel decrease in number and size of pituitary acidophils. Growth-promoting potency could be detected in plasma of protein-depleted animals but appeared to be reduced below normal levels.

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Extractable Insulin Measured by Immuno-Chemical Assay: Effect of Tolbutamide.*† (24845)

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One of the mechanisms of action postulated for the hypoglycemic effect of sulfonylureas is stimulation of pancreatic secretion of insulin. An increased content of insulin in the pancreatic vein(1), a decrease of insulin extractable from the pancreas(2), and elevated peripheral levels of insulin in plasma(3) have been reported following treatment with sulfonylureas. Interpretation of these results is complicated by the difficulties encountered with current methods for insulin assay. Bioassays of small quantities of insulin in biological fluids in general are based upon the effect of insulin on glucose metabolism(4-6). Although these methods are highly sensitive, the fact that they measure physiological effects of insulin, rather than level of hormone itself, makes them nonspecific. Since many hormones from the adrenal or adenohypophysis affect glucose metabolism, the results of these biological assays are therefore usually reported in terms of "insulin-like" activity. Methods for measuring insulin chemically by chromatography (7) and by fibril formation(8) have proved more specific, but lack the sensitivity required for most biological studies. Immuno-assay of insulin by classical hemagglutination technics has been used only for measurement of comparatively pure insulin solutions(9). We have demonstrated the highly specific nature of binding of insulin-I^{131} to antibodies in serum from diabetics requiring large quantities of insulin(10). This binding phenomenon, measured by the technic of hydrodynamic flow (11), forms the basis of a sensitive, specific assay for insulin. The use of this new method to investigate changes in extractable insulin from pancreas after administration of tolbutamide is the subject of this report. The fact that prolonged treatment with sulfonylureas results in insulin depletion in the pancreas (2) does not necessarily indicate a cause-effect relationship between the drug and the level of hormone. The concomitant long-term hypoglycemia resulting from treatment may, in itself, cause insulin depletion by preventing insulinogenesis(12). For this reason only the immediate effects of sulfonylurea were studied.

Methods. Crystalline beef-pork insulin, glucagon free, (Lot No. T-2842) was obtained from Eli Lilly and Co. Tolbutamide (Orinase®) was obtained from Upjohn Co. Insulin-I^{131} was prepared by the method described previously(10). Antisera were obtained from diabetic subjects who required more than 500 units of insulin/day. Pooled antiserum was diluted with 5% bovine serum albumin until .5 ml bound about 85% of 0.1 μg of insulin-I^{131}. Addition of unlabeled insulin in increasing quantities resulted in saturation of binding sites, reflected by decrease in percentage of insulin-I^{131} bound. This change in percentage of bound, labeled insulin with increasing concentrations of insulin forms the basis of our method of assay. To obtain a standard curve various amounts of nonradioactive insulin contained in 25 μl of 5% albumin were incubated with 0.1 ml of diluted antiserum, 10 μl (0.02 μg) of insulin-I^{131}, and 0.1 ml of 30% urea in 5% albumin‡ (pH adjusted to 8.6 with concentrated base) for 30 min at room temperature. Fifty μl of the incubation mixture was then subjected to hydrodynamic flow in barbital buffer at pH 8.6(10). In this system, free insulin stays

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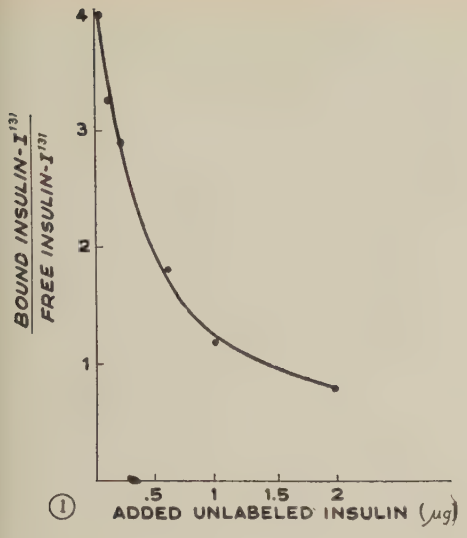
‡ Use of urea was found necessary to prevent precipitation of proteins in the acidic crude insulin solution, insoluble at higher pH. In the absence of urea about 50% of the insulin is lost during incubation by adsorption or co-precipitation with these insoluble proteins.



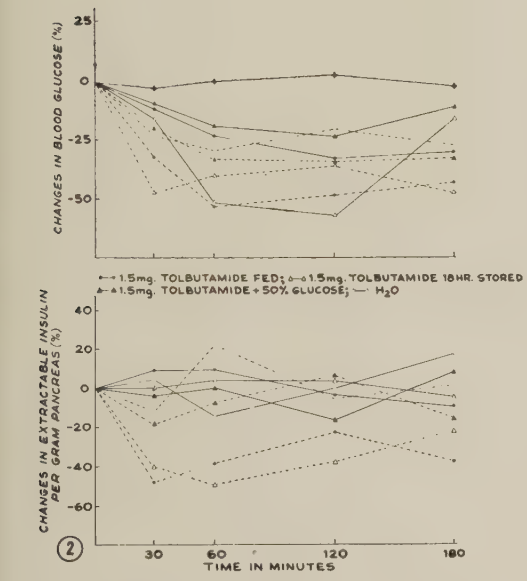


FIG. 1. Effect of concentration of unlabeled insulin on binding of insulin-I^{131} to antibodies obtained from insulin-resistant diabetics.

FIG. 2. Effect of tolbutamide on blood sugar and extractable insulin from pancreas. Solid lines, initial experiments; broken lines, duplicate experiments.

at the origin and bound insulin travels as a single peak with serum proteins. After 1 hr of flow the papers were dried, and distribution of I^{131} activity was determined by dividing the strips into 1 cm segments and counting them in a well-type scintillation counter. To obtain the standard curve, total activity in the bound fraction divided by total activity in the free fraction (B/F ratio) was plotted against concentration of added unlabeled insulin (Fig. 1). Since B/F ratio did not diminish linearly with, or as a logarithmic function of, insulin concentration, it was necessary to obtain at least 5 points corresponding to 5 different concentrations of unlabeled insulin in the incubation mixtures for determining the standard curve. The unknown crude insulin solutions were assayed simultaneously by substituting 25 μl portions of them for nonradioactive insulin-albumin solution used in the standards. Incubation and hydrodynamic flow were carried out as described above. The B/F ratio found for the unknown sample was read against the standard curve to obtain its insulin content. *Animal experiments.* 350 male mice, aged 6-8 weeks and weighing 15-18 g, either fed or starved 18 hr, were used. Only animals varying less than 10% in weight were used on a given day. The mice were given by intubation 1.5 mg tolbutamide, either in 0.2 ml water or 50% glucose solution, and were sacrificed in groups of 4 at intervals during ensuing 3 hr. An equal number of control animals, given either water or 50% glucose solution by intubation, were killed at same intervals. Mice in each group were stunned, then partially decapitated. From each animal, 0.1 ml of jugular vein blood was taken and pooled for blood sugar determination(13). The tail of the pancreas, which contains most of the islet tissue(14) and is compact and comparatively free of fat, was removed and placed in chilled saline. The excised organs from each group of 4 animals were pooled, washed for 5 min in chilled saline, blotted dry and weighed. To precipitate the protein, the pancreas (350-400 mg wet weight) was added to 4 ml of (v/v) chilled saline-10% trichloroacetic acid. After homogenization and centrifugation, the insulin was extracted from the precipitated protein with acidic alcohol (15 ml 12 N HCl diluted to 1 L with 75% ethanol) and further purified by the method of Grodsky and Tarver(7). The final precipitate of crude insulin was dissolved in 0.01 N HCl to give a volume of 0.5 ml. Other organs besides pancreas were treated in similar manner. In assaying, 25 μl of this un-

TABLE I. Assay of Extractable Insulin from Various Tissues of the Mouse.

Tissue	No. of determinations	Extractable insulin, μg/g tissue
Pancreas (tail)	12	25–42
Liver	4	.0
" + $\frac{100\ \mu g\ insulin}{g\ tissue}$	8*	85.7 (S.D. = 8.2%)
Spleen, brain, kidney, lung, heart, intestine	2 each	.0

* Refers to No. of repeated determinations of a single sample.

known insulin solution was incubated with antisera, insulin I^{131} and urea solution as described above. After B/F ratio was determined and amount of insulin in the 25 μl sample was obtained from the standard curve, insulin concentration/g of organ was equal to:

$$\frac{\text{insulin conc. of aliquot x 20 x 1000}}{\text{mg tissue}}.$$

Results. Table I records values obtained for extractable insulin from various tissues of the fed mouse. Of tissues investigated, only the pancreas contained detectable levels of insulin (25-42 μg/g tissue). The efficiency of the acid-alcohol extraction procedure is not known. When insulin-I^{131} was added to pancreas and the mixture extracted in the described fashion, only 45-55% of the label was recovered; the greatest loss occurred during first step of extraction procedure. Higher yields (74%) were obtained by Bornstein-Downie method(15), but this method was unsatisfactory because the extracted proteins were insoluble at neutral and alkaline pH's required for assay.

Recovery of insulin added to extract of liver by binding method of assay was 85.7%. The precision of the method was indicated by the S.D. of 8.2% obtained after repeated assays of a single sample.

Fig. 2 shows the effect of 1.5 mg of tolbutamide on blood sugars and extractable insulin measured in 140 fed and fasted mice. In each experiment (20 animals), blood sugars decreased 25-50% within 60 min. Addition of 50% glucose during intubation increased actual glucose levels but did not affect percentage change after administration of tolbutamide. Intubation of water alone did not affect blood sugar levels.

Changes in amount of extractable insulin after administration of tolbutamide did not correlate with the drop in blood sugar. In 5 experiments involving 100 fed or starved mice (not counting controls), insulin levels remained constant despite severe hypoglycemic response. On 2 separate occasions, once in fed and once in starved mice, a decrease in extractable insulin was observed. The associated hypoglycemia, however, was less, or no more severe, in these animals than in similarly treated mice whose insulin levels remained unchanged.

It has been suggested(16) that the effect of tolbutamide on insulin secretion can be enhanced by simultaneously giving glucose to stimulate pancreatic insulin production. Our data show that intubation of 50% glucose did not modify the effect of tolbutamide on levels of pancreatic insulin.

Discussion. Binding of insulin-I^{131} to antisera forms the basis of an assay method for extractable insulin which is chemically specific for the protein molecule, rather than being dependent on physiological action of the hormone. Steroids, growth hormone, tolbutamide and glucagon do not interfere with the assay(10). Drawbacks of the method are its complexity and the necessity for assuming that mouse insulin and the crystalline beef-pork insulin used in making the standards have the same capacity for binding to the antiserum. Nevertheless, the values of 25-42 μg of insulin/g of mouse pancreas, found in this study, are comparable to those obtained with other methods(unpublished). The actual insulin content of pancreatic tissue may be considerably higher than the reported values, since studies with insulin-I^{131} show only a 50% recovery after extraction. This recovery value cannot be used to correct for total insulin content of pancreas, however, since reportedly crystalline insulin may not have the same extraction characteristics as endogenous or crude insulin(17).

Our studies on immediate effect of tolbutamide indicate that depletion of extractable insulin in mouse pancreas is not consistently seen in conjunction with appearance of hypo-

glycemia. These findings are in line with reports that degranulation of islet cells(18) or deposition of glycogen into dorsal fat(16) does not occur after short-term treatment with sulfonylureas. Contrary to hypothesis in the latter report, the pancreatic insulin level remained unchanged when glucose was administered together with tolbutamide.

The failure to observe depletion of insulin does not necessarily exclude the possibility that tolbutamide stimulates secretion of small quantities of insulin. Because of the limitation of the method a depletion of 15-25% of total insulin content of the pancreas would not be detectable; however, such an amount represents sufficient secretion of insulin (0.7-1.0 μg) to effect a severe hypoglycemia in the mouse. Also, if secretion of insulin were followed by rapid insulinogenesis, a net change in pancreatic insulin might not occur. On the other hand, the data are compatible with the concept of an extrapancreatic action of tolbutamide. Possibly the pancreas is producing an unknown hormone with chemical structure different from that of insulin, capable of exerting a hypoglycemic effect. Tolbutamide may act on the pancreas to stimulate secretion of this hormone. The existence of such a substance, although speculative, would explain why tolbutamide activity reportedly requires a functioning pancreas but does not elicit consistent changes in content of chemically measurable insulin.

Summary. The new method for immunochemical assay of extractable insulin from the pancreas described in this report is based on the change in binding of insulin-I^{131} to insulin antibodies in the presence of varying quantities of unlabeled insulin. The method was used to study changes in extractable pancreatic insulin after administration of tolbutamide to normal mice. Although tolbutamide caused a sharp decline in blood sugar within 2 hours of administration, a consistent decrease of insulin in the pancreas was not observed.

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Interference Between Coxsackie Viruses in Mice. (24846)

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Multiple infections with enteric viruses are of considerable interest and possibly of importance to the ecology of enteric virus diseases(1). The interfering effect of Group B Coxsackie virus infection on poliomyelitis has been extensively studied. More recently, it was found that combined infection with certain Group A viruses and an attenuated polio-

TABLE I. Properties of Virus Strains Used.

Strain	No. of passage used†	Usual titer of pools‡
Coxsackie A1 #48249*	88	$10^{-8.8}$
NIH ABIV (A7) #56135	10	$10^{-5.8}$
Coxsackie A10 #50548	19	$10^{-8.5}$
" A14 #52113§	11	$10^{-7.5}$
" A21 #55161	16	$10^{-4.2}$
" B1 #49683	4	$10^{-7.7}$
" B5 #53122	14	$10^{-4.0}$

* Type collection No.

† Passages in baby mice after receipt in this laboratory.

‡ Titered in baby mice.

§ Titer in adult mice if inj. intracer. $10^{-4.8}$; if inj. intraper. $10^{-2.3}$.

virus resulted in more severe disease than either virus alone was capable of causing(2). This prompted the present study of a number of combined Coxsackie virus infections and the discovery that certain of them interfere with development of paralysis due to infection with a strain of A14 virus.

Materials and methods. Viruses. Coxsackie strains used are shown in Table I. The exceptional features of the Coxsackie A14 strain* and the ABIV virus,† a strain of Group A, Type 7, Coxsackie virus, have been described(2,3). Other members of Group A viruses seldom cause morbid effects in adult mice inoculated intracerebrally, and none, with certain exceptions, mentioned later, when injected intraperitoneally. Coxsackie Group B strains used have not induced apparent disease in adult Albany mice. All strains were cultivated in newborn mice. Ten % leg suspensions‡ were used as virus seed. They were pooled and stored at –20°C and thawed but once. Amount of inoculum was 0.03 ml (intracerebrally or intramuscularly) and 0.05 ml (intraperitoneally). The titers are shown in Table I. Young adult mice from the Albany colony weighing between 10-12 g were pooled and redistributed by random sampling. Animals were observed daily and response to infection judged by appearance and manifestation of paralysis or death. Period of observation was 3 weeks. The symptoms were frequently confirmed by histologic examination.

Results. Preliminary tests. Groups of 8 mice were inoculated simultaneously with 2 Group A viruses, either intracerebrally or intraperitoneally. Control mice received equal amount of 10% normal baby mouse leg suspension in substitution for the second virus. No unusual effect was observed. Whenever A14 was involved, many mice were severely paralyzed, while combinations of other Group A viruses showed only minor results and only if injected intracerebrally. However, the outcome was remarkably different when certain viruses were inoculated intraperitoneally at 2-day interval. In all tests in which A14 was used as challenging virus (with exception of ABIV/A14 combination), rate of paralysis was significantly lowered.

Coxsackie A10 and A14. To obtain more precise information about the time factor, 14 groups of 18 mice each were inoculated intraperitoneally with Coxsackie A10 virus and simultaneously and at various intervals challenged with A14 strain using same route of inoculation (Fig. 1). Control animals for both virus strains received 10% normal baby mouse leg suspension as substitute for second virus. Paralysis was again less frequent in the 2-day-interval group. Only one of 18 mice injected with both viruses became paralyzed, while in the corresponding A14 control group 12 of 18 mice responded. Rate of paralysis was greater in those tests in which A14 was inoculated 2½

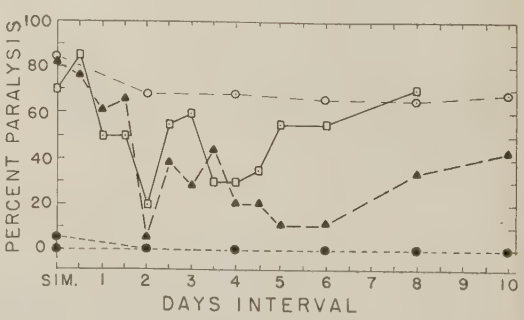

FIG. 1. Interfering effect of Coxsackie A10 and A21 on Coxsackie A14 in adult mice when inj. simultaneously and at various intervals using the intraper. route. Sim. = simultaneously. Black circles = A10 and A21 control. Dotted circles = A14 control (mean results of both tests). Triangles = A10 + A14 test. Dotted squares = A21 + A14 test.

* Received from Dr. J. H. S. Gear, Johannesburg, S. Africa.

† Received from Dr. K. Habel, Nat. Inst. Health, Bethesda, Md.

‡ In case of Group B viruses, there was no difference in titer between brain and leg suspensions.

TABLE II. Response of Adult Mice to Coxsackie A10 + A14 When Given Intraperitoneally at 2-Day Intervals.

	No. mice tested	No. mice paralyzed	Mean of reciprocal of response $\left(\frac{\Sigma 1/ti}{N}\right)^{\dagger}$
A10 controls*	100	2	.0021
A14 " * "		78	.1083
A10 + A14 test	"	21	.0237

* Both control groups received normal baby mouse leg suspension as substitute for the second virus.

† $\Sigma 1/ti$ = Sum of reciprocal of response (incubation time and paralysis). N = No. of mice tested.

to 3½ days after A10. A second phase of less paralytic response was observed when mice were given both viruses 4-6 days apart. Rate of paralysis rose gradually, but thereafter did not reach the rate of control animals at inoculation interval of 10 days. In A10 control group, which had received the virus and normal leg suspension simultaneously, one mouse was paralyzed.

The interfering effect of A10 virus on A14 at the 2-day interval was confirmed in a second experiment (Table II) in which 100 mice were used in each group. Among A14 controls, more than 3½ times as many (57% more) mice became paralyzed than among those that had received both viruses. Two of the 100 A10 control mice became paralyzed.

In both experiments, the interfering effect resulted in fewer paralyzed mice, but not in less extensive paralysis or prolonged incubation time.

The effect of route of inoculation was tested in a third experiment. Ten mice were used for each group and time interval. Mice were not protected against the effects of A14 virus given intracerebrally and intramuscular inoculation of A10 was much inferior to the intraperitoneal route.

Coxsackie A21 and A14. Twenty mice in each group were inoculated at a time. Both strains were given intraperitoneally. Although the effect of A21 on A14 (Fig. 1) was quantitatively less than that of A10, the lowest rate of paralysis was again in test group inoculated at 2-day interval. Again, the number of paralyzed mice increased in groups inoculated at 2½- and 3-day intervals and a second phase of interference was observed when the interval was 3½ or 4½ days. Later, the paralytic rate increased and no interference was evident when the interval was 8 days. Nor did interference result when other routes of inoculation were used. The outcome was similar to that with A10 virus.

Coxsackie A1 and A14. The A1 strain interfered with A14 strain as effectively as did the A10 virus. Only one of 10 mice which had received both agents at 2-day interval was paralyzed, while in the A14 control group 8 of 10 mice were paralyzed.

A7 and A14. Groups of 10 mice each were tested with both strains simultaneously and at intervals up to 6 days. The results confirmed the preliminary tests. While fewer animals were paralyzed at 1- and 3-day intervals, the number of mice responding at the 2-day interval was almost equal to those of controls.

Coxsackie B1 and A14. Preliminary tests showed a B1 strain had no effect on outcome of A14 when inoculated simultaneously or 2 days previously to the latter. In a second experiment, intervals were extended to 30 days. Ten mice were used for each group, and both viruses given intraperitoneally (Fig. 2). A first, short phase of interference appeared in test group injected at 6-day intervals, followed immediately by very sharp increase of response in mice inoculated at 7-day interval. A subsequent second phase of interference lasted through groups which received both viruses 9-12 days apart. Rate of paralysis rose in 13-15 day groups and again decreased. No paralysis occurred among mice injected 20 days previously with B1. However, 3 mice of 10 became paralyzed at 30-day-inoculation interval.

Coxsackie B5 and A14. Both viruses were given at intervals up to 15 days in the same sequence as before, using same number of mice. By taking half-day intervals whenever an effect was expected, the results more precisely revealed time range of single phases of interference.

Discussion. The experiments show that a number of Coxsackie virus infections may suppress frequency of paralysis induced by adult mouse paralytogenic strain of A14 virus.

Obviously paralysis is a convenient but limited criterion of interference and interrelationships of these agents require extensive investigation using other measures of interference. Likewise, the effect of route of inoculation needs to be explained, presumably in terms of pathogenesis of the disease in mice. Such studies might also explain the meaning of the effect of time. The experiments suggest that those viruses which do interfere do so by preventing invasion of central nervous system by A14 virus, since there has been no evidence that the evolution of paralysis was modified once it occurs. The data have, for example, been compared using Gard's method (4) in which the reciprocal of incubation interval is used without evidence that paralysis was significantly delayed.

It is of some interest that the interfering effect of Group B viruses was of longer duration than that due to Group A viruses. It was quite similar to values reported elsewhere(5) when a poliovirus rather than A14 was used as challenge. The similarity of the disease in mice caused by the A14 Coxsackie and Type 2 poliovirus matches the similarity of the interference.

Summary. 1) Young adult mice were infected simultaneously and at various intervals with Coxsackie Group A and B viruses. Under certain circumstances, representatives of both groups interfered significantly with adult-mouse-adapted A14 strain, resulting in fewer mice becoming paralyzed. 2) The grade of interference depended on time interval between injection of interfering and challenging virus, and on route of inoculation. No interference could be observed when both viruses were injected by different routes.

I am indebted to Doctor Dalldorf for his encouragement and guidance, to my wife for technical assistance, and to Miss Grace M. Sickles for many courtesies.

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Duration of Pseudopregnancy in Normal and Uterine-Traumatized Hamsters. (24847)

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Numerous studies have indicated that uterine trauma in pseudopregnant rats and certain other mammals results in prolonging pseudopregnancy and that, in rats at least, extent of prolongation is dependent on number of deciduomata induced. This study was designed to ascertain whether or not pseudopregnancy in golden hamsters may be affected similarly.

Methods. Seventy-one cyclic female hamsters, most of which had exhibited one previous pregnancy, were sterile-mated and on the fourth night thereafter, if neither vaginal nor psychic estrus was manifest, 2, 4, or 15 interrupted sutures were sewn into each uterine horn. Nightly thereafter vaginal smears were observed and willingness to mate was tested. Vaginal smears and mating responses of 52 unoperated pseudopregnant animals also were observed nightly. Pseudopregnancy in all instances was considered as having terminated when psychic estrus was again exhibited. Twenty additional animals were employed in determining the optimal day for traumatization in order to induce maximum uterine responses. At termination of pseudopregnancy the traumatized horns were examined grossly and microscopically for deciduomata. All hamsters were from the LSU colony, which has been inbred since 1943.

TABLE I. Duration of Pseudopregnancy in Normal and Traumatized Hamsters.

Group	No. of animals	Duration in days 7	8	9	10	11	12	13	14	Mean duration in days*
Normal	52		6	40	5				1	9.08 ± .84
2 loops	22		3	17	2					8.95 ± .48
4 "	22		3	14	4	1				9.14 ± .71
15 "	27	1	5	11	5	2	2	1		9.44 ±1.35

* ± stand. dev.

Results. The duration of pseudopregnancy in the several groups is indicated in Table I. Differences between the means are not statistically significant. The observed duration of normal pseudopregnancy is in agreement with that reported earlier by Deanesly(1).

At the end of pseudopregnancy gross examination of horns with 2 or 4 sutures revealed enlargements at each site of trauma. Horns with 15 sutures were massive, exhibiting many enlargements which encroached on one another. Microscopic examination revealed the enlargements to be the result principally of stromal hypertrophy, often accompanied by evidence of an incomplete, localized decidual response. In some horns the uterine lumen was much reduced and displaced; in others, large detached masses of necrotic tissue occupied the uterine canal. Vaginal smears were not always estrual at the mating terminating pseudopregnancy.

Discussion. The ineffectiveness of uterine trauma in prolonging pseudopregnancy probably is attributable to induction of insufficient competent decidual tissue. No quantitative comparisons were made of the decidual responses elicited among the experimental groups since no statistically demonstrable prolongation had been effected, and the deciduomata were insufficiently differentiated to permit quantitative study. The ineffectiveness of uterine trauma in prolonging pseudopregnancy in mice also has been reported(2).

Conclusion. An 8 to 10-day pseudopregnancy period occurs in most hamsters, the 9-day interval predominating. Hamsters rendered pseudopregnant by sterile mating do not exhibit a statistically significant prolongation of pseudopregnancy following uterine trauma, however massive, by interrupted sutures. The observations indicate species differences in luteal function in pseudopregnant hamsters as contrasted with rats.

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ACTH Releasing Activity *in vivo* of a CRF Preparation and Lysine Vasopressin.* (24848)

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This laboratory has previously reported (1) purification of a substance of hypothalamic and/or neurohypophysial origin, which stimulates release of ACTH from anterior pituitary tissue surviving *in vitro.* From evidence then available, it was proposed that the hypophysiotropic activity observed was due to a substance with characteristics of a peptide and different from any of the several known neurohumors investigated.† It was also concluded that any physiological significance of these findings obtained with simple

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† On basis of similar results obtained independently, Saffran and Schally proposed the name Corticotrophin Releasing Factor (CRF) for this substance.

in vitro methods should have to be ascertained *in vivo*, in preparations in which stress-induced ACTH release can be inhibited by pharmacological means or hypothalamic lesion. The present report will show that crude CRF preparation *fraction D*, characterized on basis of its activity *in vitro*, stimulates release of ACTH with a linear log-dose response relationship in animals with inhibition, by a hypothalamic lesion or administraton of nembutal-morphine, of the discharge of corticotrophin which normally follows upon exposure to stress.

Materials and methods. 1. *Materials.* a. *CRF Preparation* used was fraction D prepared and characterized *in vitro* according to Guillemin *et al.*(1). This particular batch (No. 300-6) of fraction D has a vasopressor activity of 0.4 U/mg when assayed against USP Posterior Lobe Reference Standard in the rat. b. *Lysine vasopressin (LVP).* A sample of purified vasopressin prepared by the method of Ward and Guillemin(2) was further purified using partition chromatography on paper with system m-cresol/H_2O(3). After elution, the lysine vasopressin (Rf: 0.82) had a specific activity of 287 U/mg confirmed on 2 subsequent 4-point assays in duplicate on lyophilized material. Assays for pressor activity were performed in the rat, against USP Posterior Lobe Reference Standard. 2. *ACTH-release in morphine-nembutal blocked animals.* 200 animals (rats, males, 175-200 g B.W., Holtzman Farms, Houston) were used. Multiple doses of CRF fraction D and of highly purified lysine vasopressin were administered intravenously according to time schedule outlined in Fig. 1. Release of ACTH was assessed by variations of concentrations of plasma free corticosteroids using the fluorometric method of Silber as modified by Guillemin *et al.*(4) using 2 ml of plasma, and also by adrenal ascorbic acid depletion method of Sayers *et al.*(5). In each experiment, whether one or multiple doses of CRF or vasopressin were tested, control animals injected with solvent were introduced in randomized fashion. Administration of nembutal and morphine, according to above schedule, was shown in preliminary experiments to inhibit the discharge of ACTH which normally follows laparotomy, cannulation of carotid artery, or injection in jugular vein. This agrees with original report of Munson and Briggs (6). 3. *ACTH-release in animals with effective hypothalamic lesion.* a. 390 animals (rats, males, 220-250 g B.W., Holtzman Farms), were used. 18 to 19 hrs. after stereotaxic placement of a lesion in median eminence of the hypothalamus (see below), all animals were tested for effectiveness of the block of stress-induced ACTH discharge as follows: they were placed in ether jar for 3 min, allowed to recover, anesthetized again 12 min later and 1.2 ml of blood taken from jugular vein exactly 15 min after beginning of stress period. Corticosteroid levels were determined immediately on 0.5 ml plasma(4). Animals with plasma corticosterone levels <20 μg/100 ml (see 7) were considered to have inhibition of corticotrophic response to stress. Twenty-four hrs. after placement of lesion, animals having shown inhibition of ACTH discharge when exposed to stress, were given Nembutal i.p. (4.5 mg/100 g B.W.)

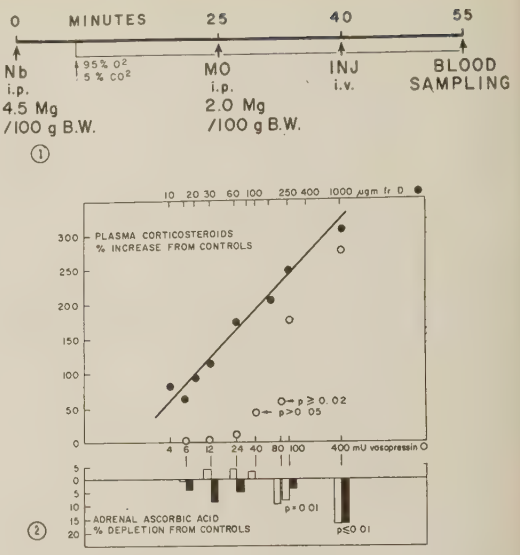

FIG. 1. Schedule of administration of nembutal and morphine, intrav. inj. and blood sampling. When indicated on diagram, animals are placed on an electric blanket at 37.5°C in a 30″ × 15″ lucite box through which a mixture of 95% O_2, 5% CO_2 is circulated at 8 l/min.

FIG. 2. Stimulation of ACTH release by CRF *fraction D* and vasopressin in nembutal-morphine blocked rats. Simultaneous measurements of plasma corticosteroid levels and adrenal ascorbic concentrations.

and 25 min. later, fraction D or vasopressin was injected in jugular vein. A sample of blood (1.2 ml) was taken from jugular vein 15 min. later for measurement of plasma corticosteroid levels. Stimulation of ACTH release by injected material was assessed by comparing levels of plasma free corticosteroids of this sample with those of earlier sample obtained after exposure to stress. The sensitivity to ACTH of the rat with effective hypothalamic lesion was determined 24 hrs. after placement of lesion according to same protocol, using 0.5, 1.0 and 1.5 mU ACTH USP Standard. b. *Hypothalamic lesion.* A minute lesion of the posterior (bulbar) part of median eminence was made, using a high frequency generator, the electrodes placed with a modified Krieg-Johnson stereotaxic instrument. The brains of *all* animals were preserved at autopsy, and reconstructions of lesion from frozen sections are available for each animal. A detailed study of lesions will be reported separately along with various physiological findings obtained in these animals. 4. *Possible potentiation of circulating ACTH by fraction D.* 24 hrs. after hypophysectomy, 24 animals (rats, males, Holtzman, 150-180 g B.W.) were given 0.5 mU ACTH USP Standard in 0.01 N HCl-saline followed exactly 60 sec later by 10 μg or 50 μg of fraction D, both substances being administered intravenously. Adrenocorticotrophic activity was assessed 15 min after injection of ACTH by measuring levels of plasma free corticosteroids(7).

Results. 1. *Stimulation of ACTH-release in nembutal-morphine "blocked" animal.* The results summarized in Fig. 2 show that injection of CRF fraction D stimulates release of ACTH with a linear log-dose response relationship. Injection of lysine vasopressin is ineffective *i.e.* does not stimulate release of ACTH, until a threshold of approximately 60-80 mU pressor activity is reached. A sudden discharge of ACTH is then observed, which seems to increase linearly with dose of vasopressin. The release of endogenous ACTH, stimulated by injection of fraction D as measured by variations of plasma corticosteroid levels, can be obtained without changes of adrenal ascorbic acid concentration when these 2 variables are measured in the same animals. Depletion of adrenal ascorbic acid occurs only upon administration of a dose of pressor activity of approximately 80-100 mU. ACTH-sensitivity of nembutal-morphine blocked rat is of the order of the 24-hr. hypophysectomized rat, when tested at multiple doses of ACTH USP standard, ranging from .025 mU to 1.5 mU.

2. *Stimulation of ACTH-release in the rat with an "effective" lesion of median eminence of hypothalamus.* The results are summarized in Table I. Of 390 animals with a lesion confirmed by reconstruction of the brain, only 107 had complete inhibition of stress-induced ACTH release according to our criteria *i.e.,* measurements of plasma corticosteroid levels. The block bore no relationship to presence or absence of diabetes insipidus. 24 hrs. after placement of hypothalamic lesion, animals with complete block of response to stress have a sensitivity to ACTH which is definitely inferior to that of the 24 hr. hypophysectomized rat; the smallest effective dose of ACTH is 1 mU *vs.* 0.1 mU for the hypophysectomized rat.

Injection of multiple doses of CRF fraction D stimulates release of ACTH, with a linear log-dose response relationship. Additional data are necessary to ascertain whether the responses at higher doses of fraction D are really on a plateau, or simply scattered about the regression line. This observation may be related to the minor resistance to ACTH of adrenals of the 24-hr. hypothalamic lesioned rat. Lysine vasopressin does not stimulate discharge of ACTH in the hypothalamic-lesioned rat until a dose of 60-80 mU pressor activity is reached. Injection of 10 or 50 μg of fraction D, 60 sec after administration of 0.5 mU of ACTH USP Standard in the 24-hr. hypophysectomized rat, did not alter (potentiate or diminish) the response of adrenals to the injected ACTH. Injection of multiple doses of fraction D (15, 30, 250 and 500 μg) in the 24-hr. hypophysectomized rat showed the material to have no direct adrenocorticotrophic activity or ACTH contamination using plasma corticosteroid or adrenal ascorbic acid concentrations as indicators of corticotrophic stimulation.

Discussion. The results would indicate

TABLE I. Effects of Various Doses of CRF Fraction D and Lysine Vasopressin (LVP) on Plasma Corticosterone Levels of Rats with Median Eminence Lesion.

No. of animals	Plasma cpd. B after stress, μg/100 ml	Amount CRF fr. D inj. in μg	Pressor equivalent in mU	Plasma cpd. B after CRF, μg/100 ml	Variations plasma cpd. B in μg/100 ml	p value‡	% variations from control level
8	10.2 ± 2.3*	20.0	8.0	16.9 ± 1.9	+ 6.6 ± 2.0†	=.01	+ 65
8	11.3 ± 1.7	40.0	16.0	26.3 ± 1.9	+14.9 ± 2.4	<.01	+132
8	10.9 ± 2.6	80.0	32.0	31.6 ± 2.7	+20.7 ± 3.0	<.01	+190
8	12.8 ± 2.1	160.0	64.0	32.9 ± 1.6	+20.1 ± 3.5	<.01	+157
5	4.2 ± 1.0	320.0	128.0	27.4 ± 1.7	+23.3 ± 2.0	<.01	+555

No. of animals	Plasma cpd. B after stress, μg/100 ml	Pressor amount of LVP in mU	Plasma cpd. B after LVP, μg/100 ml	Variations plasma cpd. B in μg/100 ml	p value‡	% variations from control level
9	11.5 ± .7*	4.0	14.5 ± 2.9	+ 3.0 ± 2.2†	>.2	0
6	11.5 ± 2.5	8.0	8.6 ± 2.0	− 2.9 ± 1.9	=.2	0
9	12.0 ± 1.2	16.0	10.5 ± 1.2	− 1.5 ± 1.7	=.4	0
5	10.9 ± 1.2	32.0	19.3 ± 3.8	+ 8.4 ± 3.3	>.05	0
9	12.0 ± 1.2	64.0	20.5 ± 2.4	+ 8.4 ± 1.9	<.01	+ 70
4	12.3 ± 1.0	128.0	26.3 ± 3.2	+14.0 ± 2.0	<.01	+114

* Stand. error of the mean. † Stand. error of the mean difference.
‡ p calculated from t on values for plasma cpd. B levels before and after CRF or vasopressin.

that a substance in fraction D, different from vasopressin stimulates release of ACTH *in vivo,* in animals with hypothalamic lesion or pharmacological blockade of the discharge of ACTH induced by stress. These results are in agreement with earlier conclusions regarding corticotrophin releasing activity *in vitro* of fraction D and vasopressin(1). The failure of McCann(8) to observe stimulation of ACTH release in a similar series of experiments with materials corresponding to fraction D seems to have been due to his using adrenal ascorbic acid depletion test as a criterion for ACTH stimulation: the same results were observed here with this method, while in the same animals definite stimulation of corticoidogenesis due to endogenous ACTH release was evidenced (Fig. 2). The difference of sensitivity of the 2 methods (see also 7) may have accounted for these divergent results, or the 2 parameters (ascorbic acid depletion *vs.* corticoidogenesis) may partake from different regulatory mechanisms(9). The dynamics of adrenal responses (ascorbic acid/corticoidogenesis) of the 24-hr. hypophysectomized rat to exogenous ACTH(7) and that of the 24-hr.-hypothalamic lesioned rat to CRF are remarkably similar. If the ratio for these responses (ascorbic acid/corticoidogenesis) were different in the normal animal when exposed to stress, the question would be raised anew(10) as to whether ACTH alone can account for the whole adrenal response upon exposure to stress. This point deserves undoubtedly attentive investigation.

The argument of McCann (in 8) on differences of specific activity of fraction D and vasopressin in stimulating release of ACTH is difficult to follow. Such comparisons are significant only when dealing with *pure* materials. Fraction D is a crude peptide mixture made by a single chromatographic separation and which, upon further purification by electrophoresis, reveals multiple components one of which only (fraction D-delta) has hypophysiotropic activity and accounts for about 1/100th of the weight of fraction D(1). No such material was available for our experiments. However, a purified CRF prepared by carboxymethylcellulose chromatography has been reported by this laboratory, more potent in releasing ACTH in nembutal-morphine blocked rat than its equal weight of lysine vasopressin(11). Comparison of respective specific ACTH-releasing activity of CRF and vasopressin will have to await availability of pure CRF. Stimulation of ACTH release by large doses of vasopressin in animal with an effective hypothalamic lesion confirming the original observations of McCann(12) is best explained by attributing inherent CRF-activity to the molecule of vasopressin. This overlapping of physiological activities (pressor,

oxytocic, hypophysiotropic) for vasopressin and CRF may be tentatively related to possibly close structural relationships between the 2 molecules(2,13). Available results do not eliminate the possibility(14) that these large doses of vasopressin may have potentiating effects on circulating ACTH and/or direct effects on the adrenal cortex.

Summary. The CRF preparation *fraction D* stimulates release of ACTH in animals with hypothalamic lesion or pharmacological blockade, at doses where pressor equivalents as pure lysine vasopressin are inactive. Hypophysiotropic activity of fraction D observed *in vitro* is therefore confirmed *in vivo* and should be attributed to a substance different from vasopressin.

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Goat Red Blood Cells in Agglutination Test for Infectious Mononucleosis.* (24849)

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Taiwan is one area of the world in which sheep red blood cells (rbc) are not readily available. Sheep are not economical animals to raise on this island because of high mortality from the liver fluke (*Dicrocoelium dendriticum*). Goats, however, are available. This investigation was undertaken to determine whether goat rbc could be substituted for sheep rbc in agglutination test for infectious mononucleosis(1). The study was designed solely to compare agglutination of goat cells with sheep cells when tested with serum containing heterophile antibody and not to evaluate the clinical significance of these reactions. The ox cell hemolysin test was also performed, since this test had been recommended as a substitute for sheep cell agglutination(2,3), and ox cells can be easily obtained on Taiwan.

Materials and methods. Serums: A total of 161 serums were tested; 61 were from patients and 100 were considered normal controls. Of the patient serums 42 came from the U.S. military dispensary, Taipei and 19 were obtained from the Univ. of Chicago Clinics (through the courtesy of Dr. Ross Benham, Director of the Clinical Microbiology Laboratory). In each case the serum had been submitted for determination of heterophile antibodies, indicating that infectious mononucleosis was being considered in the differential diagnosis. No effort was made to classify these patients by clinical criteria for diagnosis

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TABLE I. Comparison of Sheep and Goat Red Blood Cell Agglutination by 100 Serums from "Normal" Young Adults.

Rbc	<7	7*	14	28	56	112
Sheep	72	19	9	0	0	0
Goat	7	22	37	26	8	0

* Reciprocal of final serum dilution.

of infectious mononucleosis. The 100 serums used as normal controls were obtained from the serological laboratory of Taipei County. They were negative for syphilis and came from young adults. *Heterophile agglutination test*: The differential test of Davidsohn(4) was carried out. In the presumptive test the serum was heat-inactivated only and tested with both sheep and goat rbc. In the differential test aliquots of the serum were absorbed with guinea pig kidney antigen and beef red cell antigen. Each aliquot was then tested with both sheep and goat rbc. *Ox cell hemolysin test*: The method followed was that published by Mikkelsen, *et al.*(3) except that 0.2 ml amounts (rather than 0.5 ml) of serum, complement and ox cells were employed. *Source of rbc*: Sheep (*Ovis aries*) blood, goat (*Capra hircus*) blood and ox (*Bos taurus*) blood were obtained from the Taiwan Serum and Vaccine Laboratory. The only source of sheep rbc on the island is from the sheep maintained in that laboratory for experimental purposes.

Results. The results of testing 100 sera in the presumptive test with sheep and goat rbc to determine the "normal" range of agglutination are shown in Table I. Only 28 of these serums caused agglutination of sheep rbc (at 1:7 and 1:14 dilution) while most of the serums did agglutinate goat rbc in dilutions ranging up to 1:56.

Table II shows the results of testing the 61 serums from patients in the differential agglutination test with both sheep and goat rbc and in the ox cell hemolysin test. The serums are grouped according to their reaction in the presumptive test with sheep rbc. The 27 serums agglutinating sheep rbc in dilutions from 1:56 to 1:3584 in the presumptive test may be considered "positive" since absorption with guinea pig kidney antigen failed to remove the reacting antibody while it was completely removed by beef rbc antigen. These same 27 serums agglutinated goat rbc at dilutions ranging from 1:448 to 1:7168. Again absorption with guinea pig kidney failed to remove the antibody while beef rbc reduced the titer to the range found with the normal serum. Thus, the reaction in the goat cells was similar to that in the sheep cells except for a higher titer which averaged about 4-fold. These 27 serums all reacted in the ox cell hemolysin test. The next 31 serums listed in Table II showed "negative" reactions in the differential test with both sheep and goat rbc. Any antibody measured in the presumptive test (up to 1:28 with sheep rbc and up to 1:112 with goat rbc) was completely removed by guinea pig kidney and frequently not removed by beef rbc.

TABLE II. Results of Tests of 61 Serums from Patients with Possible Infectious Mononucleosis.

No. of serum c̄ same sheep rbc titer	Sheep-rbc-agglutination			Goat-rbc-agglutination			
	Presumptive test	Absorbed c̄ guinea pig kidney	Absorbed c̄ beef rbc	Presumptive test	Absorbed c̄ guinea pig kidney	Absorbed c̄ beef rbc	Ox-cell hemolysin
1	3584	3584	0	3584	3584	0	6144
6	1792	448–1792	0	3584–7168	896–1108	0–56	384–6144
6	896	224– 448	0	896–3584	448–1792	14–28	96– 768
3	448	56– 224	0	1792–3584	896–3584	14–28	192– 768
3	224	28– 112	0	896–1792	224– 896	14–28	48– 384
4	112	28– 112	0	448– 896	56– 448	0–28	48– 192
4	56	28	0	448– 896	112–1792	0–28	24– 48
2	28	0	7	56– 112	0	56	0
5	14	0	0	28– 56	0	14–28	0–nt
7	7	0	0	14– 56	0	7–14	0–nt
17	0	0	0	0– 28	0	0–14	0–nt
*	14, 14, 0	14, 0, 0	0, 0, 0	28, 28, 28	28, 14, 14	0, 0, 0	nt, 0, 0

Results are expressed as the reciprocal of final serum dilution. In agglutination test 0 = <7. In hemolysin test 0 = <6.

* Three serum with unusual low titer reaction patterns.

nt = not tested.

Again, agglutination in goat rbc closely paralleled sheep rbc agglutination except that the titer was higher with goat cells. Fifteen of these serums were tested by ox cell hemolysin and none reacted.

The last 3 serums at bottom of Table II showed a low titer reaction different from other negative ones. The titer of antibody in the presumptive test (1:14 with sheep rbc and 1:28 with goat rbc) was too low to be considered positive, but it was not removed by absorption with guinea pig kidney in 1 serum with sheep cells and in all 3 serums with goat cells. It was removed in each case by beef rbc antigen. Two of these serums were tested in the ox cell hemolysin test and failed to react.

Summary and conclusions. Sera from 61 patients showed similar reactions with both sheep and goat rbc in the differential agglutination test for infectious mononucleosis. Twenty-seven sera caused positive differential agglutination with both sheep and goat rbc and reacted in the ox cell hemolysin test. No other sera reacted positively in any of the tests. Goat cells reacted with higher dilution of serum than sheep cells with both patient serums and with 100 normal serums. A positive reaction in goat rbc was of approximately 4-fold higher titer than with sheep rbc. Within limits of these investigations it is concluded that the differential agglutination test with goat rbc and the ox cell hemolysin test gives the same laboratory information for diagnosis of infectious mononucleosis as is obtained from the sheep rbc agglutination test.

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Agglutinating Action of Heat-Inactivated Passage A Mouse Leukemia Filtrates on Mouse Red Blood Cells.* (24850)

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Since a potent mouse leukemia virus (Gross) has been developed by serial cell-free passage through newborn mice of an Ak-leukemia-derived agent(1,2), an attempt was made to determine whether leukemic filtrates containing this agent would agglutinate, or hemolyze, mouse red blood cells (rbc) *in vitro*. In preliminary tests, heated (60°C ½ hr) filtrates were also used, as controls. The surprising observation was made that fresh filtrates were with rare exceptions essentially inactive, whereas heated filtrates had consistently a distinct agglutinating effect on mouse erythrocytes. Experiments were therefore carried out to determine some of the conditions related to agglutinating action of mouse leukemia filtrates on mouse rbc *in vitro*.

Methods. Filtered (Selas 02) extracts of 20% concentration were prepared in usual manner(1-3) from C3H donors with primary, passage A(1,2) virus-induced leukemia. Part of extract was placed in ice-water at 0°C, and used within a few hours for the test. Another part of extract was heated at 55°C for ½ hour. In a few experiments, extracts were also heated to temperatures varying 40° to 60° for ½ hour. Similar extracts were prepared from spontaneous Ak mouse leukemias, from several spontaneous C3H mouse mammary carcinomas, from x-ray induced C3H leukemias, from normal organs (spleen, liver, heart, lungs, kidneys, testicles), and embryos, removed from young healthy C3H mice. *Red blood cells* were obtained by heart puncture,

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TABLE I. Agglutination of Mouse RBC by Heated (56°C ½ Hr) Filtrates from (a) Passage A Leukemic, (b) Spontaneous Ak Leukemic, and (c) Normal C3H, Donors.

Extracts from donors	No. of extracts tested	No. of extracts agglutinating in dilutions 1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Passage A leuk.	18	18	17	16	13	6	2	0
Spontaneous Ak leuk.	10	7	5	3	2	1	0	0
Normal C3H	9	5	2	1?	0	0	0	0

performed with light ether anesthesia, from young healthy C3H or C3H(f) mice. In a few experiments, red blood cells were also obtained from young, healthy Ak mice, and also from guinea pigs, and rabbits. Suspended first in physiological saline solution mixed with sodium citrate to prevent coagulation, they were washed twice briefly, with, and then resuspended (1%) in, chilled sterile physiological saline solution. Erythrocytes were used within a few hours after removal from animals' blood circulation. The extracts tested were distributed in Kahn tubes (11 x 75 mm) in serial dilutions (0.5 ml/tube); equal amount (0.5 ml) of freshly prepared 1% rbc was then added to each tube. Final concentration of rbc was 0.5%. Most tests were carried out at +4°C temperature. Several tests were carried out at room temperature (21°C), and a few in incubator at 37°C. Except for tests carried out at incubator temperature, where readings were made after 4 to 6 hours, in all other tests at refrigerator and room temperatures cells were allowed to settle overnight, and the results read next morning by the pattern method.

Results. Agglutinating action of leukemic passage A filtrates was consistent in dilutions up to 1:160 and sometimes up to 1:320 or 1:640, provided that heated (55°C ½ hr) extracts were used for the tests (Table I). Fresh leukemic filtrates had only occasionally a "questionable" agglutinating effect in either highest (1:20) concentration or, curiously, in one of the higher dilutions (1:80 or 1:160). Most fresh leukemic filtrates tested, however, were in all dilutions completely inactive on agglutination tests.

Heating of leukemic filtrates for ½ hour to 40°, 45°, or 50° was insufficient to bring out their full agglutinating potency. Heating to 55°C for 30 minutes was sufficient, and was used routinely. Extracts retained their agglutinating activity after heating at 60°C for ½ hour. In one experiment the extract was boiled 5 minutes, and still retained its agglutinating potency. Heating of leukemic filtrates to 55°C for ½ hour caused formation of a precipitate which could be readily sedimented by centrifugation at 3,000 rpm for 5 to 10 minutes. The resulting clear supernate was inactive on agglutination tests, whereas the resuspended sediment proved highly active.

C3H mouse erythrocytes were found most suitable, slightly more sensitive than those from Ak mice, and were used routinely for the tests. Guinea pig and rabbit erythrocytes were also agglutinated by heated (55°C ½ hr) leukemic extracts, but to a lesser extent.

Effect of temperature on agglutination. Agglutination tests were routinely carried out at +4°C. The heated (55°C ½ hr) leukemic filtrates agglutinated rbc also at room temperature (21°C); there was no elution of agglutinin. The tests appeared more sensitive, however, when carried out at +4°C. When a few tests were carried out at 37°C, agglutination also occurred, although in some tubes at least, it was accompanied by a slight hemolysis. Agglutinating potency of leukemic filtrate was not inactivated by leaving the extracts, prior to mixing with rbc, for several hours at room temperature. In one experiment the filtrate was kept at 21°C for 5 hours, then heated (55°C ½ hr) and finally mixed

FIG. 1. Agglutinating action of heated (55°C, ½ hr) passage A leukemic filtrate on C3H mouse erythrocytes. Results read by pattern method, looking at bottom of tubes in mirror. Cells were allowed to settle overnight at +4°C temperature. Back row (top): heated extracts agglutinating in dilutions up to 1:320. Front row (bottom): fresh filtrates; no agglutination.

with rbc. In another experiment, the filtrate was first heated (55°C ½ hr), then left at 21° C for 5 hr and finally mixed with rbc. In both experiments agglutination resulted exactly as in simultaneous controls carried out with filtrates heated and mixed with rbc immediately after preparation.

Ultracentrifugation of leukemic filtrates prior to heating did not sediment the agglutinin. In 2 experiments, passage A leukemic filtrates were centrifuged in Spinco Model L ultracentrifuge at 0°C, using Swinging Bucket rotor SW 39, at top speed 40,000 rpm (average 125,000 x g) for 45 minutes. The resulting supernate was then heated (55°C ½ hr) and mixed with rbc. In both experiments ultracentrifugation did not affect agglutinating potency of leukemic extracts. Heated supernate was active on agglutination tests as the original extract. Since leukemogenic and oncogenic activity of extracts had been previously found to be sedimented after centrifugation for only 30 minutes at 40,000 rpm using rotor SW 39(4), it appears that agglutinin is separable from leukemic virus and related oncogenic agents present in the leukemic filtrates.

Filtrates from x-ray-induced-leukemia were tested, using either C3H donors with primary x-ray-induced-leukemia (150 r, total body, 4 times, at weekly intervals), or those with passage X virus induced leukemia(5). Two filtrates were prepared from primary x-ray-induced-leukemia, and both agglutinated when tested after heating (55°C ½ hr), none when fresh. Two passage X filtrates were tested, and both agglutinated, but only after heating, in dilutions to 1:160.

Filtrates from spontaneous Ak mouse leukemia were also tested. Fresh Ak filtrates had no agglutinating effect on either Ak or C3H rbc. Heated (55°C ½ hr) filtrates agglutinated in some instances only, and in higher concentrations. Of 10 filtrates tested, 3 did not agglutinate at all (Table I).

Filtrates from spontaneous C3H mouse mammary carcinomas were tested in 3 experiments, using for extracts either single mammary carcinomas, or several pooled mammary tumors. Neither fresh, nor heated (55°C ½ hr) filtrates had an agglutinating effect on C3H mouse erythrocytes.

Fresh filtrates from normal organs (liver, spleen, heart, lungs, kidneys, testicles) from young, healthy C3H(f) mice had no agglutinating effect. Of 9 heated (55°C ½ hr) normal organ filtrates tested, 5 agglutinated in dilutions 1:20, and 2 of these also in dilution 1:40 (Table I); the remaining extracts were inactive in all dilutions tested.

A filtrate was also prepared from normal C3H embryos; no agglutination resulted when either fresh or heated (55°C ½ hr) embryo filtrate were mixed with C3H erythrocytes.

Serum neutralization. In 3 experiments, serum obtained from a rabbit that had received several weekly injections of passage A leukemic filtrates was inactivated (56°C ½ hr). Normal inactivated rabbit serum was used in a simultaneous control experiment. Heated (55°C ½ hr) passage A leukemic filtrate was first tested for agglutinating potency and the highest dilution (usually 1:80 or 1:160) which still caused agglutination was determined; the last 2 highest dilutions of leukemic filtrate which were able to cause agglutination were then used for serum neutralization tests. Serial dilutions of either immune serum (0.25 ml), or normal serum in the simultaneous control series, were then mixed with equal quantities (0.25 ml) of proper dilutions of leukemic filtrate and incubated at room temperature for 1 hour. Mouse rbc (1%) were then added (0.5 ml), and test tubes placed in refrigerator. The results were read next morning by the usual pattern method. The immune rabbit serum in dilutions up to 1:128 neutralized the agglutinating potency of leukemic extracts whereas normal rabbit serum had a neutralizing effect only in 1:4, or at the utmost 1:16, dilutions.

Discussion. It is evident from these experiments that passage A leukemic filtrates, known to induce leukemia consistently, when inoculated into suckling mice of a susceptible strain, agglutinate C3H or Ak red blood cells *in vitro*. Curiously, this agglutinating potential of the leukemic filtrates becomes evident only after extracts have been heated (55°C ½ hr), *i.e.*, after their leukemogenic potency has been essentially destroyed. It is possible

to speculate that fresh extracts contain an inhibitor which can be inactivated by heating, not unlike mouse pneumonitis virus, which was also found by Mills and Dochez(6) to agglutinate murine erythrocytes, provided that lung extracts containing the virus, had been heated to 80°C for 5 minutes prior to agglutination test. Whether the agglutinating potency of leukemic extracts is actually related to presence of the leukemic agent, however, remains to be determined. If related, the agglutinin would be separable from the agent by ultracentrifugation. The agglutinin is relatively stable, and it does not elute. For that reason, agglutination tests could be carried out either at refrigerator, or at room temperatures, although the former appeared more suitable.

Filtrates from spontaneous Ak leukemia agglutinated only in some instances, and in higher concentrations. Since it was previously observed that on inoculation tests only some of the Ak filtrates were leukemogenic(4), the fundamental question remains as to whether the agglutinating potency of some of the Ak filtrates tested could be related to their ability to induce leukemia. In 3 experiments thus far performed, inactivated (56°C ½ hr) serum from a rabbit which had received several injections of passage A filtrates in view of immunization, inhibited the agglutinating potency of passage A leukemic filtrates. Such a serum had also a partially neutralizing effect on leukemogenic potency of the extracts(5).

In control experiments, fresh or heated (55°C ½ hr) filtrates from pooled normal organs, or mouse embryos, agglutinated only in some instances and in higher concentrations. A few filtrates prepared from spontaneous mammary carcinomas did not agglutinate.

In 1947 the author observed(7,8) that filtered extracts prepared from spontaneous mouse mammary carcinomas, a transplantable mouse sarcoma, and spontaneous or transplanted mouse leukemia, exerted a destructive action on mouse red blood cells *in vitro*. Many of the extracts tested, particularly those prepared from spontaneous mouse mammary carcinomas and mouse leukemias, hemolyzed mouse red blood cells after incubation at 37°C for 2½ hours. A clumping of red blood cells was also observed, particularly when extracts from tumors were used. All tests were made in incubator at 37°C, or at room temperature. Similar results were observed with extracts prepared from human tumors(9). All these tests were made with fresh tumor filtrates.

Agglutinating or hemolytic action of mouse tumor and mouse leukemia extracts was not consistent, however. Their agglutinating and/or hemolytic potency disappeared after extracts had been kept at room temperature for only a few hours. Furthermore, agglutinating and/or hemolytic potency of these extracts was evident only in high concentrations, in most instances not exceeding 1:10 or 1:20, and only when fresh extracts were tested. Normal organ extracts only occasionally showed a similar effect(7,8).

Salaman also reported(10) that mouse tumor extracts agglutinate red blood cells; rabbit erythrocytes were most sensitive, those of mice 4 to 16 times less sensitive. Again, this potency was very labile, disappearing in extracts left for only a few hours at room temperature. Furthermore, extracts prepared from normal organs showed a similar potency. These tests again were made with fresh extracts; their potency diminished rapidly after heating.

The agglutinating action of leukemic filtrates described here differs fundamentally from that observed in our previous experiments, or those carried out by Salaman. The main difference is that the agglutinin now described requires heating (55°C ½ hr) prior to mixing with rbc, and that it is relatively stable. It is unaffected when standing for at least 5 hours at room temperature, whereas those described previously were extremely labile. In Salaman's experiments, rabbit erythrocytes were most sensitive; in our studies, murine erythrocytes were considerably more sensitive to the agglutinating action of leukemic filtrates than those from either guinea pigs or rabbits.

It was recently reported by Eddy, Rowe and their associates(11) that the "polyoma" virus agglutinated guinea pig, hamster and human erythrocytes. The term "polyoma" was given by Stewart and Eddy to the parotid tumor virus originally isolated from

Ak leukemia, and identified in our laboratory in 1953(12), and later grown in tissue culture by Eddy and her associates(13). The main differences between agglutinating property of the parotid tumor virus,† and that of leukemic filtrates, are as follows: a) the parotid tumor virus agglutinates‡ when either fresh or heated (60°C ½ hr), whereas leukemic filtrates agglutinate only after heating (55°C ½ hr); b) the parotid tumor virus elutes, and for that reason the tests must be carried out at refrigerator temperature, whereas the leukemic agglutinin described here does not elute; c) murine erythrocytes are more sensitive than those of guinea pigs to the agglutinating action of leukemic filtrates.

Summary. 1) Heated (55°C ½ hr) passage A leukemic filtrates had an agglutinating effect on mouse erythrocytes in dilutions up to 1:160, occasionally higher. Fresh filtrates did not agglutinate. 2) Agglutinin could be separated from leukemic agent by ultracentrifugation. 3) Tests were done at +4°C but could also be carried out at room temperature; the agglutinin did not elute. 4) Heated (55°C ½ hr) filtrates from spontaneous Ak leukemias agglutinated only in some instances, and in higher concentrations. Whether the agglutinating potency of some Ak leukemic filtrates is related to their ability to induce leukemia, remains to be determined. 5) Fresh or heated filtrates from pooled normal mouse organs agglutinated only in some instances, and in higher concentrations. 6) The agglutinating potency of heated leukemic passage A filtrates could be inhibited by specific rabbit immune serum to 1:128 dilution. Normal rabbit serum had an inhibiting effect only in 1:4 to 1:16 dilutions.

† This refers to *tissue-culture-grown* parotid tumor virus, which agglutinates rbc *in vitro*, and also induces high incidence of parotid, and other tumors, following inoculation into newborn mice. Curiously, however, fresh filtrates prepared *from parotid tumors,* do not agglutinate, and only occasionally induce parotid, and other tumors on inoculation tests.

‡ After this manuscript was completed, the activation of hemagglutinin in certain low-potency preparations of parotid tumor (polyoma) virus, by heating to 56°C ½ hr, was also reported (Hartley, J. W., and Rowe, W. P., *Virology,* 1959, v7, 249).

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Hyperlipemia and Hemolysis I. Interaction of Sodium Oleate with Human Erythrocytes. (24851)

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The lytic action of fatty acids on washed mammalian erythrocytes suspended in isotonic saline has long been recognized, although the precise mechanism remains speculative(1,2). Lysis of the same erythrocytes, when suspended in homologous plasma, requires significantly higher concentrations of fatty acid since normal plasma contains potent inhibitors of fatty acid hemolytic activity(3-6). Despite this marked hemolysis-inhibitory property of plasma *in vitro,* evidence has accumulated that *in vivo* hemolysis

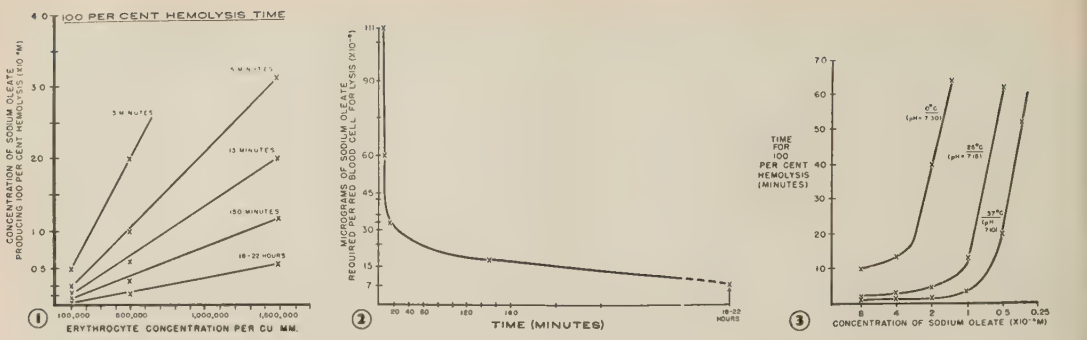

FIG. 1. Relation of human erythrocyte concentration to concentration of oleate required to induce 100% hemolysis at comparable rates at 37°C, pH 7.1.

FIG. 2. Relation of quantity of sodium oleate available per human erythrocyte (in 1.0 ml vol) to 100% hemolysis time at 37°C, pH 7.1. (Data derived from Fig. 1.)

FIG. 3. Effect of temperature on 100% hemolysis time of human erythrocytes exposed to varying concentrations of oleate. Each value represents mean of 5 determinations.

occurs during conditions associated with rapid increases in serum "free" fatty acids.* Thus *in vivo* hemolysis has been reported following alimentary hyperlipemia(8), intravenous infusions of hyperlipemic plasma or lipid emulsions(9,10), or injection of heparin into lipemic animals(11). The mechanisms underlying such *in vivo* hemolysis have not been entirely defined. The present report is the initial observation which may clarify certain intermediate hemolytic pathways.

Methods and materials. Blood of all major groups was collected from normal subjects utilizing citrate or heparin and the erythrocytes separated after 15 minutes at 2500 rpm, 0°C. Red blood cells were washed 3 times in sodium phosphate buffered saline, pH 7.15, ionic strength 0.15. The final erythrocyte suspension was adjusted by Klett-Summerson photoelectric colorimeter (filter 550 mu) so that addition of 0.5 ml of the standardized erythrocyte suspension to 2.5 ml of solution resulted in final concentration of 5×10^5 cells/cu mm. Sodium oleate (Baker) was employed as fatty acid; all solutions were prepared in phosphate buffered saline. The effect of temperature on pH of buffered oleate solutions as measured with glass electrode was as follows: 37°C, pH = 7.10; 25°C, pH = 7.15; 0°C, pH = 7.30. With certain specified exceptions, 2.5 ml volumes of fatty acid solutions (or saline controls) were utilized throughout, to which 0.5 ml aliquots of standardized washed erythrocyte suspension were added. All fatty acid solutions remained at room temperature 24 hours prior to use since hemolytic potency declined upon standing, rapidly at first, then more slowly. This phenomenon has been noted previously(12). Surface tension of fatty acid solutions was estimated by measuring rise of fluid into clean glass capillary tubes of 1 mm internal diameter; reproducibility was obtained within ± 4.2%. When this method was checked against reported measurements of temperature effect on surface tension of distilled water (13), the values agreed within ± 5%.

Results. The data presented in each experiment, summarized in Figures and Tables, are representative of similar experiments performed on human erythrocytes obtained from 5 normal subjects:

Effect of erythrocyte concentration on rate of oleate hemolysis. The time required for 100% lysis of human erythrocytes by varying concentrations of sodium oleate at 37°C, pH 7.1, varied with concentration of erythrocytes. Dervichian(14) reported similar results for horse erythrocytes suspended in lauric acid. For any fixed time interval within the ranges tested, concentration of sodium oleate necessary for 100% hemolysis bears a linear relation to concentration of erythrocytes to be lysed (Fig. 1). This finding indicates a stoichiometric interaction of oleate with hu-

* Since present evidence indicates that "free" fatty acids are not free but bound reversibly by serum proteins, the term unesterified fatty acid is preferable(7).

man erythrocytes and suggests that for 100% hemolysis at any given time interval at 37°C, pH 7.1, each erythrocyte binds (or inactivates) a fixed quantity of fatty acid.

Effect of "absorption" of oleate solutions with human erythrocytes. Studies to test the binding of oleate by human erythrocytes were performed by assaying ability of oleate solutions to lyse fresh human erythrocytes after "absorption" with aliquots from the same erythrocyte suspension. Typical results are indicated in Table I. Prolongation in hemolysis time of the supernatant solution obtained after oleate-erythrocyte contact indicates that human erythrocytes bind (or inactivate) oleate; greater binding occurs at 37°C than at 0°C and after increased contact time. The paradoxical increase in hemolytic activity of the supernatant oleate solution when contact is prolonged so that appreciable lysis of the "absorbing" erythrocytes occurs, suggests that following rupture of erythrocyte, hemolytic substances are released.

Evidence for release of hemolytic factors following oleate hemolysis. Standardized suspensions of human erythrocytes were exposed to varying concentrations of sodium oleate at 0°C, pH 7.3 for 30 seconds and spun down within 3 minutes at 3000 rpm, 0°C. The oleate supernates were set aside for assay of residual hemolytic activity, and erythrocytes washed 4 times with 3 ml aliquots of iced buffered saline. Despite such washing, 100% hemolysis ensued upon warming to 37°C, the observed rates, (Table II) correlating with those predicted from erythrocyte binding of oleate computed from residual hemolytic activity of the oleate supernates. Tests of the last 3 iced saline washings for lytic activity at 37°C were essentially negative; the final hemolyzed supernate, however, consistently lysed additional erythrocytes. Lysis rate was significantly below that of original oleate solution and was dependent upon initial oleate concentration (Table II). These findings suggest that 1) oleate binding by human erythrocytes is not dissociated appreciably by repeated iced saline washings; 2) erythrocyte binding of oleate in fixed volume increases as the initial oleate concentration is increased; and 3) following oleate hemolysis, hemolytic substances are released. Although it seemed likely that the released lytic material was oleate or products of oleate-erythrocyte interaction, it was necessary to determine whether untreated human erythrocytes contain hemolytic factors or substances that intensify hemolytic activity of oleate traces not removed

TABLE I. Binding of Sodium Oleate by Human Erythrocytes.

2.5 ml oleate solutions (2×10^{-4} M) "absorbed" with 0.5 ml standardized human erythrocyte suspensions at:		Optical density of "absorbed" oleate solutions (550 mu)	100% lysis time of 0.2 ml fresh standardized human erythrocyte suspensions added to 1 ml "absorbed" oleate solutions at 37°C† (min.)
Temp., °C	Time*		
0	0	0	7
"	1	"	10
37	0	20	45
"	.5	32	60
"	1	100	12
"	1.5	400 (100% lysis)	5
Unabsorbed control oleate solution		0	2.5

* Minutes prior to centrifugation at 3000 rpm, 0°C, 3 min.

† Mean of 4 determinations. (The longer the lysis time, the less hemolytically active oleate present. This relation is indicated in Fig. 1.)

TABLE II. Release of Hemolytic Substances after Oleate Lysis of Human Erythrocytes.

0.5 ml standardized human erythrocyte suspensions added to 2.5 ml sodium oleate of conc.:*	100% lysis time of washed red blood cells upon warming to 37°C†	100% lysis time of 0.05 ml fresh standardized human erythrocyte suspensions added to 2.5 ml of:†			
		Saline washing #			Lysed erythrocytes‡
		2	3	4	
($\times 10^{-4}$ M)	(min.)	(min.)			
8	2.0	>120	>120	>120	1.75
4	"	"	"	"	2.5
2	4.5	"	"	"	7.0
1	50	"	"	"	80

* Erythrocytes added to oleate solutions at 0°C, pH 7.3; after 30 sec., red cells spun down at 3000 rpm, 0°C, for 3 min., then washed 4 times with 3.0 ml buffered saline (pH 7.3) at 0°C and resuspended in a final volume of 3.0 ml buffered isotonic saline.

† Mean of 4 determinations.

‡ All tubes adjusted to same starting optical density with buffered isotonic saline to account for erythrocytes lost during washings.

TABLE III. Effect of Lysed Human Erythrocyte Constituents on Oleate Hemolysis.

.5 ml standardized human erythrocyte suspensions added to 2.5 ml sodium oleate (pH 7.1, 37°C) containing:		100% hemolysis time (min.)†		
		Oleate concentration ($\times 10^{-4}$ M)		
Lysate* (ml)	Isotonic saline (ml)	4	2	1
	.50	2.5	3.0	5.5
.05	.45	"	"	13
.10	.40	"	5.5	"
.20	.30	"	"	"
.50		"	6.2	30 ‡

* Lysate prepared by repeated freezing and thawing of standardized human erythrocyte suspensions followed by renewal of stroma at 4500 rpm, 0°C, 2 hr. Lysate *per se* was non-hemolytic.

† Mean of 4 determinations.

‡ When 0.50 ml lysate was added 20 sec. *after* suspension of human erythrocytes in the oleate, hemolysis time was 5.5 min.

by washing. Standardized suspensions of human erythrocytes in buffered saline were therefore lysed by repeated freezing and thawing and the stroma removed by 2 hour centrifugation at 4500 rpm, 0°C. The supernatant lysed erythrocyte constituents exhibited no detectable hemolytic activity. Table III indicates the effect of lysed erythrocyte constituents on lytic activity of sodium oleate. The findings reveal that under the defined conditions, normal erythrocyte constituents prepared by freezing and thawing retard oleate hemolysis when added to fatty acid *prior* to insertion of intact human erythrocytes (retarding effect waning as oleate concentration increases) and do not alter oleate hemolysis rate when added 20 seconds *after* oleate-erythrocyte contact. Thus the hemolytic activity observed after oleate hemolysis is probably not attributable to normal erythrocytic factors.

Quantitation of oleate required for lysis of human erythrocytes. As seen in Fig. 1, to shorten the 100% oleate hemolysis time, disproportionately greater increments of oleate are required for any given increment in erythrocytes. This relation can be expressed in terms of mean quantity of oleate required/erythrocyte for 100% lysis at any given time (Fig. 2). The quantity of sodium oleate required/erythrocyte for 100% lysis at 37°C, pH 7.1, becomes asymptotic with respect to time axis; a mean minimum of approximately 7×10^{-9} μg of sodium oleate/erythrocyte/1 ml is required for hemolysis to occur at a rate detectably greater than that of control untreated erythrocytes. Conversely, under the same conditions, when greater than 111×10^{-9} μg of sodium oleate are available/erythrocyte/1 ml, lysis time is no longer shortened detectably.

Influence of temperature on oleate hemolysis. Temperature variations markedly alter oleate hemolysis rates (Fig. 3). At least 2 mechanisms appear to be involved. Cooling from 37°C to 0°C not only decreases the rate (and/or amount) of erythrocyte oleate binding as indicated by Table I, but also slows the rate at which oleate induces lysis after erythrocyte binding. Thus when standardized suspensions of human erythrocytes were exposed to sodium oleate at 0°C for periods too brief to permit lysis and then washed 5 times with iced buffered saline, those aliquots warmed to 37°C consistently lysed more rapidly than controls held at 0°C. The role of increase in pH of oleate solutions from 7.1 to 7.3 upon cooling from 37°C to 0°C in retarding erythrocyte binding of oleate and slowing hemolysis after binding was not determined.

Effect of non-specific enzyme inhibitors on oleate hemolysis. To determine whether oleate hemolysis involved an enzymatic reaction, several non-specific enzyme inhibitors were tested. Addition of 0.01 M (final concentration) fluoroacetate, fluoride, or azide failed to alter the rate of oleate hemolysis of human erythrocytes. Sodium cyanide, however, added to 1×10^{-4} M sodium oleate (25°C, pH 7.2) delayed 100% lysis of 5 standardized human erythrocyte suspensions from a mean of 3.5 minutes (control) to: 12 minutes at 5×10^{-2} M, 20 minutes at 5×10^{-3} M, and 6 minutes at 5×10^{-4} M of cyanide ion.

Role of surface tension reduction on oleate hemolysis. Addition of sodium oleate to buffered isotonic saline lowered surface tension, reduction at any given temperature increasing progressively with increasing oleate concentration. It seemed possible that oleate hemolysis rates might be a function of such surface tension alterations. Fig. 1, however, indicates that within the ranges of oleate and erythrocyte concentrations tested, the 100%

hemolysis time can be maintained constant despite varying fatty acid concentration (and hence varying surface tensions) by altering quantity of erythrocytes added. This suggests that the lowered surface tension to which erythrocytes are *initially* exposed upon introduction into oleate solutions is not an important determinant of 100% lysis time. That surface tension lowering plays no important role even during the latter phases of oleate hemolysis was suggested by experiments in which standardized human erythrocyte suspensions were added to increasing dilutions of 50 x 10^{-2} mg sodium oleate starting with 2 ml solutions; despite an 8-fold dilution and a progressive rise in surface tension, the 100% lysis time at 37°C, pH 7.1, remained constant.

Discussion. The initial step in lysis of human erythrocytes by oleate at pH 7.1 and with concentrations below 8 x 10^{-4} M appears to involve binding of fatty acid by the red blood cell. This was first suggested by the observation that the quantity of oleate required to induce 100% hemolysis within any given time period maintained a linear relation to number of erythrocytes to be lysed. Subsequent studies indicated that contact of normal washed human erythrocytes of all major blood groups with sodium oleate consistently resulted, prior to any detectable hemolysis, in removal of the oleate from solution. Such removal was not proved by chemical analysis, but was indicated by reduced hemolytic activity of the supernatant oleate solution. The alternative possibility that oleate was rendered hemolytically inert is untenable in view of subsequent observations. Moreover, recent evidence demonstrates that human erythrocytes are capable of binding radioactive sodium oleate-I^{131}(15) and sodium palmitate-1-C^{14}(16). Within concentrations employed, the lowered surface tension of oleate solutions did not influence hemolysis rates.

Once human erythrocytes are exposed to oleate under conditions permitting binding of hemolytic quantities, the union is firmly established; lysis cannot be checked by repeated iced saline washings. Hemolysis now proceeds at pH 7.2 $\pm$ 0.1 as a function of the quantity of oleate bound and of temperature. The temperature effect is complex. A decrease from 37°C to 0°C slows oleate hemolysis by at least 2 mechanisms: 1) erythrocyte binding of oleate is slowed (and/or reduced), and 2) lytic action of oleate following binding is retarded. Since the quantity of oleate binding is a determinant of hemolysis rate, it might be expected that once erythrocyte oleate binding capacity is saturated, the 100% hemolysis time (at any given pH and temperature) no longer would decrease as oleate concentration increases. This indeed occurs and is attained with a mean of approximately 111 x 10^{-9} μg (36 x 10^{-17} moles) of sodium oleate/erythrocyte at pH 7.1, 37°C. Although this evidence is not conclusive for erythrocyte saturation at these levels, the values may be compared with the mean of 5.3 x 10^{-17} moles of binding sites/human erythrocyte reported for sodium palmitate-1-C^{14} determined at pH 7.45, 23°C(16). The mean minimum quantity of sodium oleate required/human erythrocyte for lysis at rates detectably faster than control untreated red cells at pH 7.1, 37°C is approximately 7 x 10^{-9} μg.

Ability of cyanide ion to retard oleate hemolysis was of interest. Whether this was related to enzymatic interference is unknown; however other non-specific enzyme inhibitors —fluoroacetate, fluoride, and azide—failed to alter oleate hemolysis rates in final concentrations of 0.01 M.

Following lysis of erythrocytes binding oleate, hemolytic factors were released. Since such factors could not be detected from normal erythrocytes lysed by repeated freezing and thawing, it seems probable that oleate (or some product of oleate-erythrocyte interaction) is liberated following oleate hemolysis. Assuming the released hemolytic material to be oleate, the mechanism of release is unknown. Fatty acid probably enters the red blood cell after initial attachment to the surface membrane(1,16); since red cell ghosts bind fatty acid as avidly as intact cells(16), it is tempting to speculate that the lytic factor released following oleate hemolysis represents that fraction of oleate transferred *into* the erythrocyte. Although the hemolytic activity of such released material was always significantly lower than that of the original

oleate solutions, it is apparent that oleate possesses the potential of inducing a decelerating hemolytic chain reaction under proper dynamic circumstances—*i.e.*, when erythrocytes binding oleate lyse after moving into areas populated by normal erythrocytes.

Summary. 1. The initial step in human erythrocyte lysis by oleate at pH 7.2 ± 0.1 and concentrations below 8 x 10^{-4} M involves erythrocyte-oleate binding; oleate binding by a standard number of erythrocytes in a fixed volume is a function of oleate concentration, temperature, and contact time. 2. Once lytic quantities of oleate are bound, hemolysis proceeds at pH 7.2 ± 0.1 as a function of quantity bound and of temperature. Lysis cannot be checked by repeated iced saline washings. A mean minimum of approximately 7 x 10^{-9} μg of sodium oleate is required/erythrocyte at 37°C, pH 7.1 for lysis at rates detectably faster than controls; the sodium oleate binding mechanism appears saturated with a mean of approximately 111 x 10^{-9} μg/erythrocyte. 3. Lowered surface tension of oleate solutions does not influence hemolysis rates under conditions tested. 4. Cyanide retards oleate hemolysis; other nonspecific enzyme inhibitors fail to do so. 5. Following oleate hemolysis, hemolytic substances are released; these probably represent oleate or products of oleate-erythrocyte interaction. It is suggested that oleate possesses the potential of inducing a decelerating hemolytic chain reaction.

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Comparative Effects of Glycogen and Antigen-Antibody Reactions on Serotonin and Histamine in the Rabbit. (24852)

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In rabbit blood, serotonin (5-hydroxytryptamine) and histamine are present in, or bound to the platelets. When antigen is added to blood from a sensitized rabbit, clumping of platelets and leucocytes occurs, and serotonin as well as histamine is released from platelets (1). During anaphylaxis in the rabbit, similar changes occur. In addition, a decrease occurs in whole blood concentration of these amines(1). This decrease is secondary to disappearance of platelets from circulating blood. The rapid fall in number of platelets and leucocytes in blood is a consistent finding during hypersensitivity reactions in animal species including dogs, monkeys, guinea pigs, and rabbits(2,3,4). In humans, sensitive to

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ragweed, intradermal injection of ragweed antigen produces a decrease in number of platelets in peripheral blood(5). Recent evidence shows that during anaphylaxis in the rabbit, platelets are trapped in the lung but not in other organs or tissues(6). When antigen-antibody complex precipitate, suspended in saline, is added to normal rabbit blood *in vitro* or is injected intravenously into normal rabbits, platelet-leucocyte clumps are formed and serotonin and histamine are released. *In vivo,* a marked decrease in whole blood serotonin and histamine occurs, accompanied by trapping of platelets in lung but not in other tissues(6). These findings suggest that direct action of antigen-antibody complex itself is the main causative factor for changes in platelets, serotonin, and histamine which occur during anaphylaxis. Clumping of platelets and leucocytes, associated with disappearance of these blood elements from peripheral circulation has been implicated in Schwartzman (7) and Arthus(8) reactions in rabbits. Platelet-leucocyte clumps have also been found in hearts of patients dying from acute rheumatic fever or rheumatic carditis(9). Glycogen, after intravenous injection into rabbits and dogs, caused rapid clumping and disappearance of platelets and leucocytes from the circulation similar to antigen-antibody reaction(4). Extracts from parasites, *Ascaris lumbricoides* and *Echinococcus granulosus* (hydatid fluid), also have the ability to produce the same blood changes. The active principle in these preparations is polysaccharide in nature(4). Glycogen *in vitro,* however, did not cause release of histamine, and *in vivo* was thought to distribute platelets and leucocytes equally among various tissues. Dextran, on the other hand, when incubated with rabbit blood *in vitro* does release large amounts of histamine from the platelets(10). *In vivo* in rats, dextran produces a typical anaphylactoid response(11). The present study was undertaken to determine the effect of glycogen on serotonin, histamine, and platelets in rabbits both by *in vitro* and *in vivo* technics. The results are compared with those obtained by using an antigen-antibody complex precipitate, in addition to those results found during complete antigen-antibody reaction.

Materials and methods. Male white rabbits (2.5 kg) were used. Ten were sensitized to horse serum as previously described(1). For *in vitro* studies, blood was obtained by cardiac puncture; for *in vivo* studies, blood was obtained by means of plastic tube inserted into carotid artery. Heparin or EDTA (disodium ethylene-diaminetetraacetate, 10% by volume of a 1% solution in n-saline) was used as anticoagulant. Siliconized glassware was used in handling blood. After death of rabbits, their lungs were immediately excised, pressed between porous paper to remove excess blood, and homogenized in 0.1 N HCl. Purified glycogen preparations from 3 different commercial sources were tried and all gave identical results. The antigen-antibody complex was precipitated from blood serum of rabbits sensitized to horse serum and given an additional 3 ml booster injection intraperitoneally of this antigen 3 weeks before blood was drawn(6). The precipitate was washed 3 times with cold saline and suspended in saline. Incubations with rabbit whole blood were carried out as previously reported(1). Serotonin and histamine analyses in whole blood, plasma, and tissues were done by the method of Weissbach *et al.*(12).

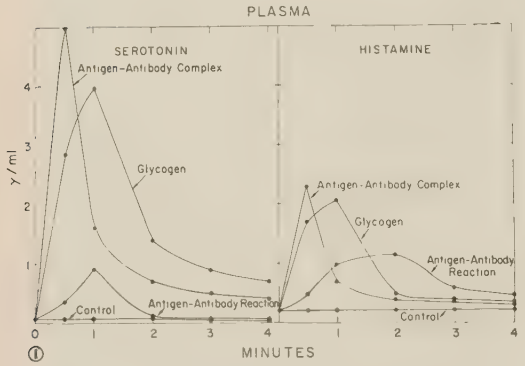
Results. Both glycogen and antigen-antibody complex release serotonin and histamine *in vitro* from rabbit platelets, and similar to release of these amines when horse serum is added to blood from rabbits sensitized to this antigen (Table I). For these *in vitro* studies, heparin was used as anticoagulant. When EDTA was used as anticoagulant the release of serotonin and histamine was inhibited in all cases. Previous work by Humphrey and Jaques(13) showed that addition of purified antigen and antibody to normal rabbit platelets suspended in normal plasma released serotonin and histamine. Removal of calcium ion from plasma inhibited this reaction.

In vivo, the effects of glycogen and antigen-antibody complex after intravenous injection into non-sensitized animals were similar to those produced by intravenous injection of antigen into sensitized rabbits. Serotonin and histamine were released into the plasma

TABLE I. *In Vitro* Release of Serotonin and Histamine.*

Agent	No. determinations	γ/ml plasma (avg values) Heparin anticoagulant Serotonin	Heparin anticoagulant Histamine	EDTA anticoagulant Serotonin	EDTA anticoagulant Histamine
Antigen-antibody reaction (1:100 horse serum in n-saline)	10	4.0	3.1	.3	.4
Antigen-antibody complex	4	5.0	3.2	.4	.4
Glycogen	6	5.5	3.5	.4	.5
n-Saline	10	.3	.4	.2	.2

* Plasma serotonin and histamine were determined after incubation of rabbit whole blood 30 min. at 37°C with 50% by volume of above agents, using heparin or EDTA as anticoagulants. For antigen-antibody reaction, blood was taken from rabbits sensitized to horse serum. Blood from normal rabbits was used for other agents. Amount of antigen-antibody complex for each experiment was precipitated from 1 ml of rabbit serum. Final dilution of glycogen was 1.3 mg/ml.



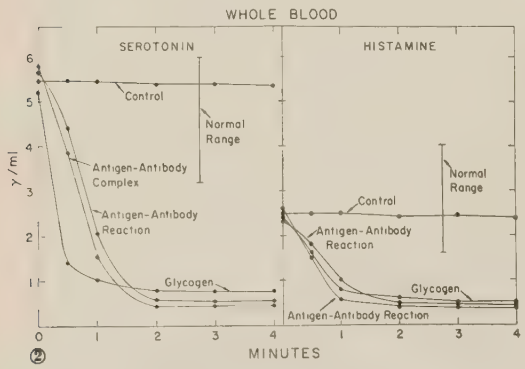


FIG. 1. Blood plasma concentration of serotonin and histamine was determined after intrav. inj. of each agent as follows: antigen-antibody reaction, 2 ml/kg of horse serum into previously sensitized rabbits; antigen-antibody complex, total precipitate from 13 ml of rabbit serum was used for each experiment and inj. into normal rabbits; glycogen, 100 mg/kg; and for normal controls, 2 ml/kg of n-saline. Five animals were used for each agent; only 2 were used with the antigen-antibody complex. Each point is avg value.

FIG. 2. Whole blood serotonin and histamine were determined on samples of blood, prior to centrifugation for plasma, used in Fig. 1.

TABLE II.

Agent	Lung serotonin and histamine,* γ/g (avg values) Serotonin	Histamine
Antigen-antibody reaction	21.5	14.3
Antigen-antibody complex	20.0	13.5
Glycogen	13.0	9.0
n-Saline	2.6 (1.2–4.6)†	4.5 (3.0–6.0)†

* Lung content of serotonin and histamine was determined in rabbits to obtain data for Fig. 1 and 2. Animals were killed exactly 4 min. after inj. of each agent.

† Normal range.

(Fig. 1), whole blood serotonin and histamine content was reduced (Fig. 2), and lung concentration for these amines was increased (Table II). The latter 2 effects were attributed to occlusion of platelet-leucocyte clumps or emboli in the lung. Other tissues, including heart, brain, intestine, and liver from these rabbits, did not show any significant difference in amount of serotonin and histamine compared to same tissues from untreated animals, except for a slight increase in serotonin in the liver.

Discussion. The results show a marked similarity between the effects of antigen-antibody complex and glycogen on rabbit platelets and leucocytes, and in release of serotonin and histamine from these platelets. The mechanisms involved appear to be the same. Other substances such as peptone(14) and trypsin(15) have been reported to cause a decrease in circulating platelets and leucocytes when injected into rabbits and dogs. Initial

observations, however, show that total results with these agents are different from those found with glycogen in this study.

Prior work with dextrans indicated that ability to release histamine from rabbit blood was dependent upon molecular size of the dextran(10). The same factor might apply to glycogen and its ability to release serotonin and histamine. The nature of end groups in the glycogen molecule, and alterations in its structure formed as a result of isolation and purification process, may also produce some of the properties in the polysaccharide needed to cause the effects found.

The distinct importance of platelets and leucocytes during anaphylaxis in the rabbit should be recognized. The role of these blood elements during allergic reactions in other animal species and in man could be of equal importance. In addition, the possibility that glycogen, or a polysaccharide-like material, may be involved in hypersensitivity processes needs further investigation.

Summary. When glycogen is incubated with normal rabbit blood, both serotonin and histamine are released from the platelets. EDTA inhibits this release. Intravenous injection of glycogen into rabbits produces a marked fall in whole blood concentration and a rise in lung content of serotonin and histamine. These results are secondary to trapping of platelets in the lung. In addition, both serotonin and histamine are released into the plasma. All of these changes are identical to those obtained when antigen is injected intravenously into a sensitized rabbit or when an antigen-antibody complex precipitate is injected intravenously into a normal rabbit.

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Sources of Excess Taurine Excreted in Rats Following Whole Body Irradiation.* (24853)

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Excessive urinary excretion of taurine has been observed in rat and man in the 24-hour period following exposure to whole-body x-irradiation(1-4). This taurine response appears to be an extremely sensitive and early indicator of radiation exposure, being detectable at doses as low as 35 r(4) and as early as 3 hours post-irradiation(2). Urinary taurine excretion increases semilogarithmically with roentgen dosage in the range between 0 and 250 r. Above this degree of radiation, taurine response does not increase(2). The magnitude of urinary excretion of taurine is a valuable index of survival in rats exposed to LD_{50} dose of x-ray(1). Since taurine is one of the major end products of SH oxidation

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(5), excessive excretion of taurine following irradiation might suggest an increase in rate of oxidation of this grouping. Barron *et al.* (6) have shown SH-containing enzymes especially sensitive to inactivation by x-irradiation, and various amino-SH compounds exert protective action against radiation death(7). These observations suggest that alterations in SH metabolism may play a role in cellular damage induced by x-irradiation. Thus determination of the source of additional urinary taurine excreted following exposure to whole-body x-irradiation may be of some importance in elucidation of metabolic changes occurring in the rat immediately following exposure to ionizing radiation. Taurine exists free in organs of many animals(9). On an absolute weight basis, striated muscle contains more than 75% of total body taurine of a rat(9,10). Taurine concentrations in rat organs remain remarkably constant following fasting, adrenalectomy, hypophysectomy, gonadectomy, estrogen treatment(9) and dietary procedures designed to deplete bodily stores of endogenous SH-compounds(11). On the other hand, urinary taurine levels are dependent upon diet, being decreased significantly by diets low in organic sulfur(14) and by concomitant administration of substances such as bromobenzene and cholic acid which divert cysteine into special metabolic pathways(13). The present work was designed to determine the source of urinary taurine excreted in response to whole-body irradiation.

Materials and methods. Isolation and determination of taurine from tissues and urine was done by the method of Awapara(8). Three to 4-month-old rats (Sprague-Dawley strain†), weighing 250-300 g were used and divided into 2 groups of 12 each. Group I was fed Rockland pellets and water *ad lib.* Group II was fasted 3 days and then maintained on protein-free, cholic acid-supplemented diet for 10 days prior to irradiation procedure. Protein-free diet was as used by Awapara(11)‡; cholic acid diet was prepared by incorporating 8.16 g of cholic acid into each kg of protein-free diet. The L-cystine diet contained 4.67 g/L-cystine/kg of protein-free diet. In each group, 6 rats were irradiated while the other 6 served as non-irradiated controls. Irradiation procedure was as follows: Twenty-four hours prior to irradiation all food, but not water, was removed from metabolic cages in which the rats were kept singly, and a "control" urine was collected from each rat during this 24-hour period. Then the rats were placed in irradiation cages of wire mesh. Each cage accommodated 3 rats in separate compartments. Radiation factors were: 250 Kv; 15 ma; 0.5 mm Cu and 1 mm Al filters: 50 cm T SD; 40 cm^2 field; 102 r mm to field. Three rats were irradiated at a time, each receiving 600r. Controls were handled similarly but not irradiated. Following irradiation all animals were returned immediately to metabolic cages and urine collected for a second 24 hour period. Rats in Group I, irradiated and non-irradiated, were sacrificed after 24 hours by stunning, and organ samples obtained immediately, weighed, frozen (dry ice), and stored at 4°C for subsequent analysis. Following completion of first part of the experiment, rats in Group II, which had served as non-irradiated controls, were removed from the protein-free-cholic acid diet and given a diet in which L-cystine replaced cholic acid as supplement to basic protein-free diet. These animals were maintained on the new diet for 10 days; then one-half of the group received 600r whole-body irradiation while the other half served as controls. Pre-irradiation control 24-hour urines and post-irradiation 24-hour urines were again collected.

Results. A. *Tissue taurine analyses.* Tissue concentrations of taurine prior to and following radiation are shown in Table I. Radiation did not cause significant alterations in taurine concentrations of tissues studied.

TABLE I. Tissue Levels of Taurine in Rats Exposed to X-radiation 24 Hr Prior to Analysis.

	μm taurine/g wet tissue			
Treatment	Ilio-psoas	Spleen	Thymus	Liver
Controls	13 ±.6†	7.7 ± 1.4	24 ± 2.1	.57 ± .1
Irrad.*	13 ±.8	7.2 ± 1.2	20 ± 1.8	.75 ±1

* As described under *Methods.*
† Means and stand. errors.

† Obtained from Holtzman Co., Madison, Wis.

‡ Obtained from Nutritional Biochemicals Co.

TABLE II. Effect of Radiation and Diet on Urinary Taurine Excretion.

Diet	No. of rats	Radiation dosage (r)	Control 24 hr urinary taurine excretion, mg/24 hr	2nd 24 hr taurine excretion, mg/24 hr	"P" values*
A. Normal (Rockland pellets)	6	0	36 ± 2†	41 ± 2	
B. *Idem*	6	600	36 ± 1	82 ± 2	<.01
C. Protein-free + cholic acid	6	0	5.58± .46	5.61± 1.20	
D. *Idem*	6	600	4.04± .31	11.01± 1.92	.02
E. Protein-free + L-cystine (following protein-free + cholic acid	3	0	12.2 ± 3.1	6.3 ± 1.9	
F. *Idem*	3	600	11.7 ± 4.4	16.9 ± 2.7	.1

* "P" values calculated for comparisons between irradiated and control animals with respect to increased urinary taurine.

† Means and stand. errors.

While tissue taurine concentrations do not decrease following irradiation, the net weight loss of these tissues is not great enough to account for the urinary taurine excretion(9,10). Apparently the source of taurine excreted following irradiation is not preformed tissue taurine, but rather excessive formation of taurine from precursors. Additional analyses of other organs were not necessary, since striated muscle alone has sufficient taurine content to account for the quantity excreted after irradiation(9). B. *Urinary taurine excretion.* I. *Rats on stock diet.* In irradiated rats maintained on a Rockland pellet diet, the second (*i.e.*, post-irradiation) 24 hour urines showed an increase of 46 ± 2 mg of taurine over the control 24-hour collection (Table II). In non-irradiated controls the second 24-hour urines showed an increase of 5 ± 3 mg of taurine over control 24-hour urines. The difference in increase between irradiated and controls is significant ("p" value <0.01).

II. *Rats on a protein-free cholic acid-containing diet for 10 days.* Irradiated animals in this group showed an increase, from control 24-hour collection to second 24-hour collection, of 6.97 ± 1.94 mg of taurine. Non-irradiated controls showed an increase of 0.03 ± 1.38 mg of taurine ("p" value = 0.02). Since the protein-free, cholic acid-containing diet reduced significantly the urinary taurine response to irradiation (Compare A and C, Table II), and since Awapara(11) has shown that this diet does not alter taurine content of tissues, it appears that lack of urinary taurine response in animals on this diet was due to relative absence of a requisite taurine precursor in the form of a sulfur-containing compound. In view of this, those non-irradiated animals which had been maintained on protein-free, cholic acid-containing diet were placed on protein-free, L-cystine-containing diet, to assess whether presence of sufficient amount of sulfur-containing amino acid would restore the urinary taurine response to irradiation. However, irradiated animals which had been transferred to the cystine-containing diet showed a questionably significant increase in taurine excretion (Table II, compare, Exp. E and F; "p" value = 0.1). These results may have several explanations: 1) These animals had previously been in negative N balance by maintenance on a protein-free, cholic acid-containing diet. The second protein-free diet contained only enough L-cystine to satisfy minimal nitrogen requirements and maintain the rats at constant weight. In this condition of relative protein deficiency, (each rat having lost about 30 g on the protein-free cholic acid-containing diet) dietary cystine may have been channeled into tissue protein and away from those metabolic pathways leading to taurine precursors. Thus, the normal marked urinary taurine response to irradiation was not seen. A corollary of this point might be that the period of L-cystine supplementation was too brief. 2) The possibility exists that methionine is the major source of taurine produced following irradiation. This would be predicated on the existence of as yet undescribed pathway from methionine to taurine. This possibility is indicated by the demonstration that intravenous injection of methionine

sulfoxide preceding or accompanying cholic acid ingestion produces an increase in taurine excreted as taurocholic acid(12). It is possible that methionine is converted in part to methionine sulfoxide by irradiation. 3) Although there is no definite evidence that cholic acid feeding produces liver damage and thus may interfere with production of taurine from its precursors, this possibility is not excluded and would explain the data obtained.

Summary. 1. X-irradiation does not alter concentration of taurine in muscle, spleen, thymus or liver tissue of rats exposed to x-radiation 24 hrs. prior to study. However, during this period, there is a marked increase in urinary excretion of taurine. Thus preformed tissue taurine does not appear to be the source of excess taurine excreted following irradiation. 2. Rats maintained on a diet designed to cause a depletion of sulfhydryl compounds (cholic acid feeding) have a significantly reduced urinary taurine response to whole-body irradiation. 3. When rats maintained on sulfhydryl-depleting diet were given subsequently an L-cystine supplemented diet, the lowered urinary taurine response to irradiation was not restored to normal.

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Vascular Reactivity as Influenced by Acetazolamide, Dichlorophenamide and Mercaptomerin.* (24854)

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Chlorothiazide (Diuril)† has been reported to exert an antihypertensive effect(1). However, a mechanism for this effect has not been clearly demonstrated. Previous studies have shown that chlorothiazide decreases arterial pressure responses to injections of norepinephrine, epinephrine and isopropyl-norepinephrine in the dog(2). Since it has been observed that renal effects of chlorothiazide on water and electrolyte excretion bear a similarity to the mercurial diuretics(3) and that mercurials may have an antihypertensive action(4), it was of interest to test the effects of a mercurial diuretic on vascular reactivity. In addition, the effect on vascular reactivity of acetazolamide (Diamox) and dichlorophenamide (Daranide),† diuretics which inhibit carbonic anhydrase, was determined.

Methods. Fifty-three experiments were performed on adult mongrel dogs anesthetized with sodium pentobarbital (30-35 mg/kg) and vagotomized. Arterial blood pressures were taken with a mercury manometer and recorded on a smoked drum. The experimental procedure followed in obtaining the controls and administering the drug was similar to that described previously(2). Acetazolamide, 10 mg/kg was administered in a priming dose intravenously, followed by continuous infusion of 10 mg/kg for 30 minutes. Dichloro-

* Aided by USPH grants.

† Diuril and Daranide, supplied through courtesy of Merck, Sharp and Dohme Research Labs., Merck and Co., Inc.

TABLE I. Effect of Acetazolamide, Dichlorophenamide, and Mercaptomerin on Vascular Reactivity.

Dose (μg/kg)	Control: No. of animals	Control: Before infusion (mm Hg)	Control: After infusion (mm Hg)	Control: Change	Drug: No. of animals	Drug: Before infusion (mm Hg)	Drug: After infusion (mm Hg)	Drug: Change	Level of significance*
Effect of acetazolamide on vascular reactivity									
A. Pressor responses to norepinephrine									
.05	9	17.3	16.7	– .6	9	15.2	9.0	–6.8	<.05
.10	9	24.1	22.7	–1.4	9	16.6	11.4	–5.2	"
B. Depressor responses to isopropylnorepinephrine									
.05	7	9.8	9.7	– .1	9	22.1	9.9	–12.2	<.01
Effect of dichlorophenamide on vascular reactivity									
A. Pressor responses to norepinephrine									
.05	9	17.3	16.7	– .6	8	13.1	9.8	–3.3	>.05
.10	9	24.1	22.7	–1.4	8	16.1	12.7	–3.4	"
B. Depressor responses to isopropylnorepinephrine									
.05	7	9.8	9.7	– .1	8	19.2	10.4	–8.8	<.01
Effect of mercaptomerin on vascular reactivity									
A. Pressor responses to norepinephrine									
.05	7	14.5	13.6	– .9	13	16.5	11.4	–5.1	<.001
.10	7	18.3	17.1	–1.2	13	22.3	15.9	–6.4	"
B. Depressor responses to isopropylnorepinephrine									
.05	7	9.8	9.7	– .1	8	16.1	10.2	–5.9	<.05

* Considered significant if $P < .05$.

phenamide, 20 mg/kg was given as a priming dose and 20 mg/kg infused for a 30 minute period. Mercaptomerin (Thiomerin), 2.5 mg/kg was given intravenously as a priming dose followed by continuous infusion for 30 minutes in a dose of 3.0 mg/kg. Responses in all animals were determined immediately after infusion of the diuretic with the exception of mercaptomerin; these responses were obtained 30 minutes after cessation of the infusion since no changes were noted in preliminary experiments until diuresis began with this compound. Base pressures during evaluation of the responses following each diuretic differed less than 10 mm Hg from control levels. Animals treated identically to the experimental groups with the exception that no diuretic agent was present in the glucose solutions served as controls.

Results. The results obtained are illustrated in Table I. Acetazolamide infusion decreased pressor response to norepinephrine and depressor response to isopropylnorepinephrine. These changes occurred in all animals tested and were significant. In contrast to acetazolamide, dichlorophenamide did not decrease vascular reactivity in response to norepinephrine though decreasing depressor response to isopropylnorepinephrine.

Mercaptomerin infusion reduced pressor responses to norepinephrine and depressor responses to isopropylnorepinephrine in every experiment.

Determinations of plasma sodium concentrations before and after administration of the 3 diuretics did not show significant changes. Preliminary experiments in which urinary excretion of sodium and potassium were measured indicated that acetazolamide and dichlorophamide produced increases in these electrolytes comparable to that observed with chlorothiazide. Excretion of sodium and potassium 30 minutes following cessation of mercaptomerin infusion likewise caused an increased electrolyte excretion paralleling that noted with chlorothiazide.

Discussion. The results obtained indicate that the ability of chlorothiazide to decrease vascular reactivity as measured by its ability to decrease responses to injected norepinephrine and isopropylnorepinephrine is not unique for this diuretic. The results support the concept that increased sodium excretion plays a dominant role in the diminished vascu-

lar reactivity since the common property of all the agents studied is the capacity to increase electrolyte excretion, primarily sodium. The failure of dichlorophenamide to alter pressor response to norepinephrine remains obscure.

The similarity of the changes in responses resulting from the several diuretics raises a question whether a singular intrinsic antipressor action is associated with chlorothiazide. Although the mechanism is not known, it is conceivable that the antihypertensive action of chlorothiazide may be due to: a) a direct effect upon peripheral vascular smooth muscle to alter its state of contraction, or b) a shift in sodium and potassium across the cell membrane resulting in a change in tonus of the vascular system, or c) its diuretic and saluretic action to alter plasma volume and electrolyte balance. Evidence available at the present time would suggest that the diuretic and saluretic action plays the most important role. Independent of changes in volume, excretion of large amounts of sodium may play a role in the antihypertensive properties of a diuretic, since low sodium diets will decrease blood pressure and increase the effectiveness of ganglion blocking drugs in hypertensive patients(5). Recently it has been reported that both chlorothiazide and mercurial diuretics appear to have an antihypertensive action that may be potentiated by a decrease in body sodium(4). In addition, an increase in electrolyte excretion associated with a diminished plasma volume has been observed to decrease the dosage of ganglionic blocking agents required in hypertensive patients(6). Results obtained in this study lend support to changes in electrolyte excretion as being a dominant factor. In each case, no change in response was noted until an interval of time had elapsed when diuresis was established. The possibility that a simultaneous decrease in plasma volume may have been an important factor can not be ruled out since measurements of this parameter were not made.

Summary. The effects on the vascular responses to norepinephrine and isopropylnorepinephrine induced by 3 diuretic agents, acetazolamide, dichlorophenamide and mercaptomerin were determined. Acetazolamide and mercaptomerin diminished vascular responses to these catechol amines similarly to the decrease previously reported with chlorothiazide administration. It is considered probable that the effect on vascular reactivity resulting from these agents is due to augmentation of sodium excretion.

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Interactions of Human Sera and Spinal Fluids with Human Brain Antigen and Antibrain Rabbit Serum. (24855)

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Investigations concerning both immunological differentiation of body organs (*e.g.*, 1,2) and possible existence of "autoantibodies" to human organs have been reported(3,4,5,6). Gadjusek(7) searched for "autoimmune" antibodies in sera of patients with various diseases and found positive complement fixation reactions in 3% tested with liver antigens and

in 10% tested with kidney antigens. Yeh, *et al.*,* have differentiated by precipitin test in human sera circulating protein from various organs of the body. Wide variations in quantity circulating were found. It might be hypothesized that in neurological disease, brain antigens may circulate in the serum. In the present study the possible presence of antibodies to human brain in sera and spinal fluid of various hospital populations and evidence concerning presence or absence of circulating brain antigens in sera and spinal fluids of these populations were investigated by means of precipitin formation. The results reported here constitute only a beginning in this type of study.

Materials and methods. Preparation of antigen. A portion of frontal lobe of brain from a 70-year-old male, 5 hours after death, was stored at –20°C. A 12 g portion, composed of grey and white matter, was homogenized in cold acetone, centrifuged, the acetone removed, and the residue resuspended in 150 cc of saline. *Immunization of rabbits with human brain.* Intraperitoneal injections were given twice weekly to white, male rabbits (5-7 lbs) starting with 1 cc of homogenate and increasing the dose by 1 cc a week until 5 cc were given in final fifth week. Before mixing with antiserum the same antigen homogenate was cleared by centrifuging at 4°C at 3000 rpm for 15 minutes. Sera obtained by cardiac puncture of 8 immunized rabbits were pooled. A precipitate was observed at 1024-fold dilution of this antiserum when the latter was mixed with antigen solution. Serum was obtained from (1) a group of 128 patients from the general medical and surgical service,† not having any known neuropsychiatric disorder. (2) A random group of 46 patients from neuropsychiatric wards, with various acute and chronic mental disorders, of which 2 were acute schizophrenia. (3) Group of 29 patients with schizophrenia, severe type. (4) Group of 41 patients with paresis, but negative serology. Sera from third group were collected before ataractic drug therapy and for 6 consecutive weeks after drug administration. Cerebrospinal fluid was obtained from 10 patients, 2 of which had neuropsychiatric illness and 2 had multiple sclerosis. Using adequate controls, microprecipitin tests were set up by mixing 0.2 cc of antiserum and 0.2 cc of brain antigen, human serum (diluted 1:4), or spinal fluid, incubating 1 hr. at 37°C, storing 18 hrs. at 4°C, centrifuging, and examining for precipitate.

TABLE I. Precipitin Formation with Human Serum *vs* Brain Antigen.

Type of patient	No. in series	No. positive	Highest dilution of serum giving a precipitin
Medical & surgical	128	3	16, 32, 8
Random	45	0	0
Schizophrenic	29	1	8
Paretic	41	1	80

Results. When serial dilutions of human serum were mixed with human brain solution (Table I), 3 individuals out of 128 general medical and surgical cases and 2 patients out of 115 neuropsychiatric cases gave a positive precipitin reaction, in dilutions of human sera ranging from 8 to 160 fold. A precipitate still resulted with sera drawn twice or thrice from these positively reacting patients, whose diagnoses were schizophrenia (1 case), paresis, (1 case), mild arthritis, (1 case), alcoholism (2 cases).

When various dilutions of antibrain rabbit serum were mixed with sera of human subjects, a precipitation resulted in one out of 45 random neuropsychiatric patients (2%), in 3 instances out of 29 severe schizophrenic patients (10.4%) and in 13 instances out of 41 paresis patients (31.7%), (Table II).

TABLE II. Precipitin Formation with Antibrain Rabbit Serum and Human Serum.

Type of patient	No. in series	No. positive	Highest dilution of antiserum giving a precipitin
Medical and surgical	128	0	0
Random NP	45	1	640
Schizophrenic	29	3	320, 320, 5120
Paretic	41	13	160 (2 cases) 320 (4 ") 640 (2 ") 1280 (3 ") 2560 (2 ")

* In Press, *Arch. Int. Med.*

† Furnished through courtesy of Dr. J. L. Garey, Chief, Clinical Labs.

TABLE III. Reactions of Cerebrospinal Fluid.

Type of patient	No. in series	Spinal fluid mixed with human brain antigen — No. giving precipitin	Antibrain serum mixed with CSF — No. giving precipitin	Highest dilution of antiserum giving a precipitin
Non-neuropsychiatric	6	0	6	8 (2 cases) 16 (4 ")
Random neuropsychiatric	2	0	2	8, 16
Multiple sclerotic	2	0	2	8, 32

The sera from all severe schizophrenic patients were secured before ataractic drug or placebo treatment and each week for 6 consecutive weeks thereafter. Sera from 3 of these patients gave a positive precipitin reaction for all 7 specimens of each case. A wide range of antiserum dilutions was tried and led to clear precipitates from 80-fold to 5120-fold (Table II).

The sera of 13 of 41 paretic patients showed a strongly positive reaction at various antiserum dilutions; 6 sera tested a week later remained positive.

Of 10 spinal fluids tested, none reacted with precipitin formation when mixed with brain antigen, but all gave a definite precipitin when mixed with antibrain serum (Table III), regardless of disease state of patient.

Conclusions. Rabbit antiserum to a saline soluble component of human brain, when mixed with spinal fluids and with sera from patients with various illnesses, gave a precipitate with all spinal fluids and with 17 out of 244 human sera. These positively reacting sera were from 4 schizophrenic and 13 paretic patients. On the other hand, human brain antigen mixed with human serum gave a precipitate in 5 out of 244 cases. The 5 sera were from one arthritic, one paretic, one chronic schizophrenic and 2 alcoholic patients. The few sera reacting positively show that this is not a general flocculation phenomenon, but whether immune systems actually related to brain are being tested here, is the subject of further study. The relatively high number of paretic patients giving a positive reaction with antibrain serum may have a bearing upon this. These results may provide a lead for further investigation of brain disease.

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Reactions of Stuart Factor and Factor VII with Brain and Factor V. (24856)

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Several workers have shown that a potent prothrombin conversion factor forms on incubation of factor V (labile factor, Ac Globulin, proaccelerin) with serum and brain extract (1-4). This product can be sedimented by high speed centrifugation(1) and will, for convenience, be referred to as "extrinsic thromboplastin." The factor in serum which takes part in formation of this product was believed to be factor VII (stable factor, proconvertin, SPCA). The one-stage prothrombin time is prolonged in congenital deficiency of Stuart factor (SF) as well as congenital deficiency of factor VII so that it now appears

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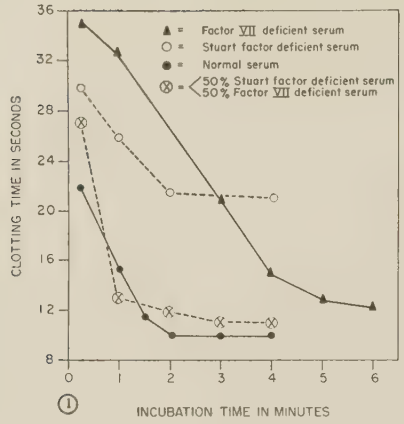
probable that SF is also involved. It is unlikely that PTC (factor IX, Christmas factor) plays a role since the one-stage prothrombin time is normal in congenital deficiency of this factor. There is, however, evidence that another factor named prephase-accelerator may be involved(5). The purpose of our work is to delineate the actions of PTC, factor VII and SF when incubated with brain and factor V. No attempt has been made to study the role of other factors such as "prephase-accelerator" in this reaction.

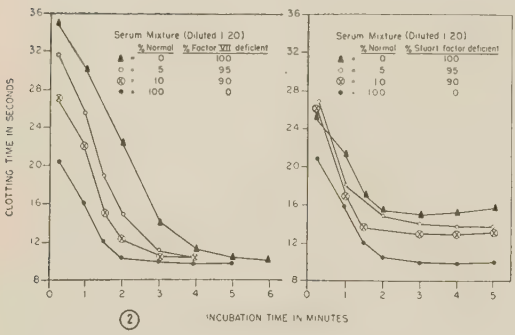
Materials and methods. Whole blood was collected in glass tubes and allowed to clot and to remain at 28°C for 24 hours before separating the serum. Plasma was obtained by adding 9 parts whole blood to 1 part 3.8% trisodium citrate and centrifuging at 3000 rpm. Plasma and serum were stored at –20°C in small aliquots and aged 2 to 3 months at time of experiments. SF-deficient plasma and serum were obtained from patient (R.S.), previously reported(6), with congenital deficiency of SF. Factor VII-deficient plasma and serum were obtained through courtesy of Drs. Harold A. Wurzel, University of Penn. and C. L. Johnston, Jr., University of N.C. from patient with congenital deficiency of factor VII; some studies on this patient have been previously made(7). *Factor V* was prepared from human blood by method of Biggs and Macfarlane(8). The *veronal buffer* (pH 7.2) used in reaction mixtures was prepared by method of Owren(9). Dilution of reagents was carried out with physiological saline. In experiments to be described 0.2 ml factor V, 0.2 ml rabbit brain (DIFCO) diluted 1:50, 0.2 ml buffer and 0.2 ml test serum diluted 1:20 were mixed in this order. Exactly 10 seconds after addition of serum, 0.2 ml of 0.025 M $CaCl_2$ was added and a stop watch started. At subsequent intervals 0.1 ml aliquots were removed from incubating mixture and added simultaneously with 0.1 ml 0.025 M $CaCl_2$ to 0.1 ml plasma substrate and clotting times recorded. All experiments were carried out in water bath at 37°C. In experiments in which mixtures of various sera were used, the individual sera were first diluted 1:20 with normal saline before mixing.

Results. Factor VII-deficient and SF-deficient sera were separately incubated with factor V, brain extract and calcium chloride and coagulant activity of each incubating mixture was then determined using normal plasma substrate (see Methods). The results (Fig. 1) show that in absence of SF, maximum activity or yield of "extrinsic thromboplastin" (reflected by shortest clotting time) was less than that of normal control. This experiment appears to show that factor VII deficiency affects rate of formation rather than yield of "extrinsic thromboplastin." There was a decrease of coagulant activity of normal incubation mixture (not shown in Figure) after minimum substrate clotting time (maximum yield) was attained; in such systems maximum yield of "extrinsic thromboplastin" in absence of factor VII was usually slightly less than that of normal control. In other test systems this apparently inhibitory phase was not seen during experiment and factor VII-deficient serum always produced normal yield. In all experiments, however, in which factor VII-deficient serum was used instead of normal serum, there was a delay in attaining minimum clotting time. A mixture of equal parts of SF and factor VII-deficient sera gave an essentially normal curve (Fig. 1) although the normal minimum was never quite reached. PTC-deficient serum gave normal result in respect of both rate and yield, and no further studies were carried out on reaction of this factor with brain.

Mixtures of SF-deficient or factor VII-deficient serum with normal serum in varying proportions were then prepared and tested as above. It was found (Fig. 2) that relatively small amounts of normal serum produced significant although not complete correction. It should be noted that in this particular experiment, yield of "extrinsic thromboplastin" in absence of factor VII was normal.

Effect of substitution of plasma deficient in factor VII or SF for normal plasma substrate. In preceding experiments, although normal substrate containing both factor VII and SF was used, an abnormal result was always obtained if one of these factors was excluded from incubation mixture. If normal serum was included in the reaction mixture, the results obtained using a substrate deficient in either SF or factor VII were identical to those obtained using normal plasma. When





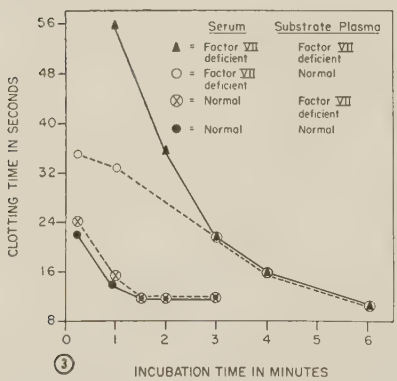


FIG. 1. Effect of substituting factor VII or SF-deficient sera for normal serum in incubation mixture containing factor V, brain and calcium using a normal plasma substrate.

FIG. 2. Effect of addition of small amounts of normal serum to factor VII or SF-deficient sera in formation of "extrinsic thromboplastin." Normal plasma was used as substrate.

FIG. 3. Effect of using factor VII-deficient plasma as substrate. Incubation mixture contained either normal or factor VII-deficient serum.

SF-deficient serum was substituted for normal serum in the incubation mixture, clotting times of SF-deficient plasma substrate were longer than those obtained using normal plasma substrate but in both instances low yields were obtained.

When factor VII-deficient serum was substituted for normal serum in the incubation mixture, the initial clotting times were longer with factor VII-deficient plasma substrate than with normal plasma substrate but the same minimum clotting times were eventually obtained (Fig. 3). SF-deficient plasma substrate gave results similar to normal plasma substrate when only factor VII was omitted from the incubation mixture.

Discussion. These results are understandable if factor VII and SF participate in a reaction with factor V and brain, producing an "extrinsic thromboplastin" which subsequently reacts with prothrombin to form thrombin. If this is what really occurs, SF and factor VII would no longer be required once "extrinsic thromboplastin" is fully formed. If, however, one of these factors is excluded from incubation mixture, presence of deficient factor in the substrate would compensate by promoting formation of "extrinsic thromboplastin" in substrate clotting tube; such substrate clotting times would be expected, of course, to be more prolonged than when all factors are present in incubation mixture.

In our experiments there is an unavoidable carrying over of factor VII and SF into substrate clotting tubes. However, when factor VII is omitted from incubation mixture but included in substrate, time required for maximum yield is same as when this factor is excluded from both substrate and incubation mixture. This finding suggests that factor VII is required only for formation of "extrinsic thromboplastin" and not for subsequent reaction of "extrinsic thromboplastin" and prothrombin. Although the yield of "extrinsic thromboplastin" is greater when SF is omitted from the incubation mixture but present in substrate than when it is absent from both substrate and incubation mixture, it should not be inferred that SF is required both for action of "extrinsic thromboplastin" on prothrombin and for formation of "extrinsic thromboplastin." It has already been men-

tioned that this greater yield would also be expected on the hypothesis that SF is required solely for "extrinsic thromboplastin." The work of Flynn and Coon(1) suggests that this hypothesis is correct. These workers sedimented "extrinsic thromboplastin" by centrifugation and then washed it by suspension in saline and recentrifugation; they showed that this washed material was active in converting purified prothrombin to thrombin in presence of calcium. The method of preparation of prothrombin was such that it is unlikely to have contained SF. However, this evidence is not entirely conclusive for some free SF not utilized in the initial formation of "extrinsic thromboplastin" might have been adsorbed onto this complex.

When factor VII is excluded from both incubation mixture and substrate clotting tubes in thromboplastin generation test, normal yield is obtained, and there is also normal rate of evolution. Therefore, the finding that absence of factor VII from incubation mixture and substrate tubes affects rate of "extrinsic thromboplastin" formation although having little or no effect on yield is interesting. SF is essential for "intrinsic thromboplastin" generation, primarily affecting yield, so that finding that this factor also primarily affects yield of "extrinsic thromboplastin" was not surprising.

Summary. Previous work showing that factor V, serum, brain and calcium react together to form an active prothrombin conversion factor ("extrinsic thromboplastin") is confirmed. The active components in serum in respect of this reaction include both factor VII and SF but not PTC. SF appears to influence yield while factor VII primarily determines rate.

Addendum. Although SF appears to affect yield rather than rate of formation of both "extrinsic" and "intrinsic" thromboplastin formation, work of Fisch and Duckert (*Thromb. Diath. Haem.,* 1959, v3, 98) appearing since this paper was submitted indicates that SF acts as an enzyme in "intrinsic thromboplastin" formation.

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An Attempt to Recover WEE from Nasal Mites of Sparrows. (24857)

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Bird mites collected from various species of birds harbor the virus of Western Equine Encephalitis (WEE)(1,2,3), and some investigators have shown that transmission of St. Louis encephalitis virus occurs when infected mites were allowed to feed on susceptible chickens(4,5). Inasmuch as no experiments on nasal mites were reported in the literature an attempt was made to recover WEE virus from inoculated sparrows and nasal mites harvested from them.

Materials and methods. Adult English sparrows of both sexes were captured from roosting places at night when flight activity was at a minimum. These birds were banded and bled from the heart for pre-inoculation neutralization test (reference sera); inoculated subcutaneously with 0.1 ml of WEE virus containing 140 chick LD_{50}; and turned loose in screened flight cage approximately 8′ x 12′ x 6′. The WEE virus (3742-c2) was isolated from a pool of *Culex tarsalis* mosqui-

toes collected in July 1954 at Greeley, Colo., and had been passed 2 times in day-old chicks. Forty-eight hours after inoculation, the birds were again bled from heart to establish whether or not WEE virus was in the circulating blood. Some sparrows did not survive this second bleeding so nasal mites were harvested from nasal pasages for inoculation into susceptible chicks. Wet chicks were used as indicator of presence of virus and also in neutralization test as described by Chamberlain *et al.*(6). Twenty days after inoculation, sex determination was made after they were bled from heart. These blood samples were tested for presence of virus and neutralizing antibodies. Nasal mites of genera *Ptilonyssus* and *Speleognathus* were collected and pooled for inoculation into "wet" chicks. Mites were prepared for inoculation by grinding with pestle in mortar with a small amount of sterile alumdum and 5 ml of a 10% normal buffered horse serum. This material was centrifuged at 1500 x g and the supernate removed for inoculation. Because of limited amount of blood obtained on first bleeding, sera were screened at one dilution only (against 12 chick LD_{50}). Following exsanguination at 20 days postinoculation, a screening was made at 2 levels, 150 chick LD_{50} and 1650 LD_{50}, again because of limited amount of sera.

Results. There was a total of 489 *Ptilonyssus* mites and 245 *Speleognathus* mites recovered from nasal passages of 44 sparrows. Although 40 of 44 had circulating WEE virus in the blood, none passed into the gut of mites at detectable levels using a very sensitive test animal (wet chicks). Those birds which did not have viremia had preinoculation neutralizing indices of greater than 12 with one exception, and this bird had both virus and antibodies. All inoculated birds tested, *i.e.*, 36 of 44, developed neutralizing indices of greater than 1650 after 20 days with one exception and this was greater than 150 but less than 1650.

Sex distribution of 30 females and 13 males, with one sex unrecorded at autopsy, did not demonstrate special susceptibility or resistance to either mites or WEE virus even though 3 males of the 13 had no mites and only 1 of 30 female sparrows was free of mites.

Discussion. Nasal mites which feed from epithelium of mucous membranes of nasal passages of sparrows probably are not vectors or reservoirs of WEE virus. The experiment described here adds evidence to agree with the conclusions of Reeves *et al.*(7), Chamberlain and Sikes(8) and Sulkin *et al.*(9) that ectoparasitic mites are not important reservoirs or vectors of encephalitis viruses.

There appeared to be excellent correlation between presence of WEE antibodies and failure to find virus after inoculation. It is unfortunate that precise levels could not be determined on the small amount of sera from each bird. As might be expected, those birds which were successfully infected developed antibodies. Quite by chance one bird was found to have both antibodies and virus present.

If it were possible to remove these mites and infect them by direct inoculation into the gut and return them to an unparasitized bird host, perhaps more information might be gathered.

Summary and conclusion. 1. Twenty days after 44 English sparrows were each inoculated with 140 chick LD_{50} amount of WEE virus, the collected nasal mites were devoid of virus using Chamberlain's "wet" chick method of isolation. 2. Nasal mites of the English sparrow are probably not important vectors or reservoirs of Western encephalitis virus.

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Existence of Fatty Acid Peroxides in Normal Blood and Tissues of Man and Animals.*† (24858)

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It is well known that fatty acid peroxides are formed in the presence of hemoglobin and other heme compounds(1), and there is evidence suggesting *a* tocopherol as an *in vivo* inhibitor of unsaturated fatty acid oxidation catalyzed by hematin compounds(2). However, it is not unlikely that fatty acid peroxides may be formed normally in small amounts from unsaturated fatty acids in animal body and may have a role in metabolism. Many methods will detect fatty acid peroxides in pure solution. Of these the thiobarbituric acid method (TBA) of Kohn and Liversedge(3) is positive in extracts of most animal tissues, but negative in blood. The indophenol dye method and thiocyanate method described by Glavind and Hartmann(4) are negative in normal tissues and in blood. It has been claimed that fatty acid peroxides can be detected in blood with methods based on liberation of iodine from potassium iodide (5,6). In spite of many inferences that fatty acid peroxides may be present normally in the animal body, few workers have been willing to conclude that this is so(5,6,7). In this paper we shall present evidence that fatty acid peroxides are present normally in both blood and tissues.

Methods. We found that sensitivity of the TBA reaction can be increased markedly by presence of 9.6×10^{-4} M ferric chloride. Control experiments with esters of linoleic, linolenic and arachidonic acids showed that autoxidation does not take place under conditions of the reaction. Using a method developed for these conditions, we found much reacting material in blood of man and many species of animals and in most normal and abnormal tissues. However, it was apparent that much of the reacting material was not fatty acid peroxides, but substances which behave like acetaldehyde, which gives the reaction in presence of this concentration of iron. A study was made of the effect of varying concentrations of iron on the TBA reaction. In Fig. 1 it is seen that in alcohol-ether solutions of partially oxidized polyethenoid fatty acids almost maximum color is attained at 1.2×10^{-4} M ferric chloride. In acetaldehyde only about 10% of maximum color is attained at this concentration of iron. In alcohol-ether extracts of blood and tissues TBA values obtained at 1.2×10^{-4} M are about 43 and 79%, respectively, of those obtained at 9.6×10^{-4} M ferric chloride. This indicates presence of acetaldehyde-like material in both blood and tissues which agrees with results of other studies. Blood apparently contains a larger amount of the contaminating substances than tissue. It appeared that 85% of the fatty acid peroxides and only about 10% of the acetaldehyde substances would react at 1.2×10^{-4} M iron. To obtain more evidence, especially in the case of blood, that unsaturated fatty acids exist partially in the oxidized peroxide form, the fatty acid fractions of blood and tissues were isolated under anaerobic conditions. Methods used were indophenol dye and thiocyanate methods of Glavind and Hartmann and the unmodified TBA method in absence of iron, since acetaldehyde-like material is not detected under these conditions. Experiments were performed on fresh normal human blood and on liver excised from living rats anaesthetized with ether. Air was excluded at all steps using nitrogen from heated copper coil and the entire procedure was conducted as rapidly as possible. The fatty acid fraction was isolated by the method of Stoddard and Drury(9) in which the acids were finally precipitated from aqueous acid solution in the cold. Peroxides were not detected by any of the 3 methods in fatty

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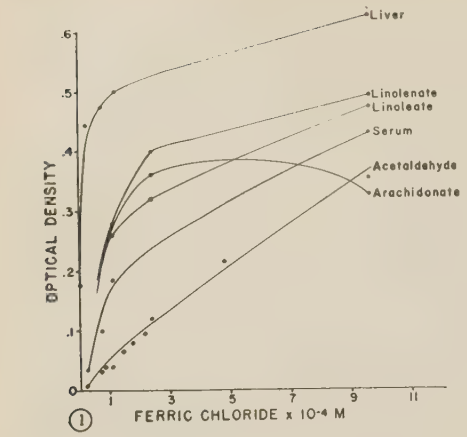

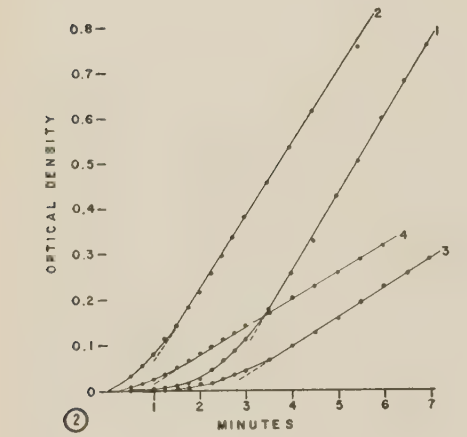

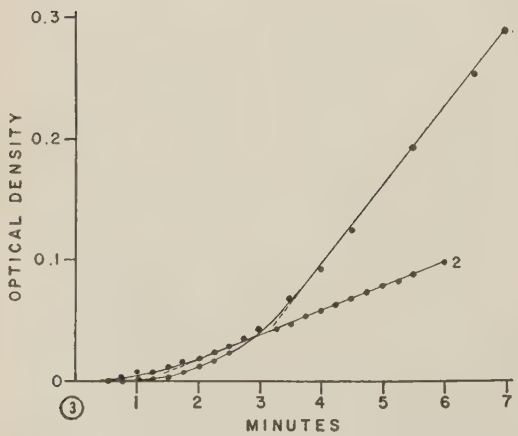

FIG. 1. Effect of varying concentrations of ferric chloride on TBA reaction.

FIG. 2. Effect of linoleate peroxide on lipoxidase-linoleate system. Curve 1, control with saline; 2, same with 0.002 μM linoleate peroxide; 3, control with 0.05 ml of 4% bovine albumin; 4, bovine albumin plus 0.002 μM linoleate peroxide. Induction period is indicated at point of origin of dotted line on curves.

FIG. 3. Effect of blood plasma on induction period of lipoxidase-linoleate system. Curve 1, control with bovine albumin; 2, test curve for 0.04 ml plasma instead of albumin.

acids isolated from blood. On the other hand, peroxides were detected in fatty acids isolated from tissues by all 3 methods. However, only about 4-18% of the quantity apparent in original tissue was recovered in the precipitated fatty acids. The results obtained in control experiments with mixtures containing partially oxidized polyethenoid fatty acids were similar, and only about 4% of the reaction was recovered in precipitated acids. Some of the reactive material remained in filtrates from fatty acids of both tissue and in control experiments, but there was still a loss of more than 50% in each case. Stearic acid added to blood and tissues was recovered quantitatively in other control experiments performed similarly except that acids were determined in the usual way by titration with standard alkali.

To show that fatty acid peroxides are present in blood, and that failure of total isolated fatty acids of blood to yield positive reactions with the 3 methods tested was due to low concentration of fatty acid peroxides and to losses encountered during isolation, another method was tried. Haining and Axelrod(8) showed that induction period which occurs during lipoxidase catalyzed oxidation of fresh sodium linoleate, can be abolished by about 5 x 10^{-3} μmoles of linoleate peroxide. Since this effect of fatty acid peroxides appeared specific, it seemed a good method to use for demonstration of these substances in blood. Fresh blood was transferred directly from a syringe to ice cold test tube containing oxalate. Nitrogen gas was bubbled through the tube, and plasma prepared in an atmosphere of nitrogen. The procedure of Haining and Axelrod was followed in detail. Fig. 2 shows values of optical density at 234 mμ in the system of lipoxidase-linoleate. Curves 1 and 2 are saline control and test curve with 0.002 μmoles of linoleate peroxide. Curves 3 and 4 are control with bovine albumin and test curve of albumin plus 0.002 μM of peroxide. Despite the fact that presence of albumin di-

minishes values of optical density, the induction period in its presence is approximately the same as in its absence. Fig. 3 shows the same system for a bovine albumin control and a test curve containing 0.04 ml of plasma but no added peroxide. It is evident that 0.04 ml of the plasma reduces the induction period approximately by half. This experiment was repeated several times on this plasma with the same result, and similar results were obtained in several other plasmas. From this effect on lipoxidase-linoleate system one can calculate the amount of fatty acid peroxides in blood, since 0.001 μmole is required to reduce the induction period of the system in half. The value of 2.5 μmoles of fatty acid peroxides/100 ml calculated in this way compares with other values of fatty acid peroxides estimated in 36 human blood serums from normal subjects and patients with a variety of clinical conditions, by the TBA method with 1.2×10^{-4} M ferric chloride. Values of TBA reaction calculated in terms of μmoles of linoleate peroxide from the value of 30,000 for the extinction coefficient at 234 mμ for completely peroxidized linoleic acid reported by Holman (10) yield a range of 8.6 to 35/100 ml. The values calculated from indophenol dye equivalent(4) yield a range of 12.4 to 50.7 μmoles of fatty acid peroxides/100 ml.

Similar calculations of fatty acid peroxides in 52 samples of rat tissues were made from results of the TBA method with 1.2×10^{-4} M ferric chloride. Calculation from extinction coefficient at 234 mμ of oxidized linoleic acid gives a range of 1.86 to 4.86 μmoles of fatty acid peroxides/g of fresh tissue. Calculation of TBA values from analysis of linoleate peroxide with indophenol dye gives a range of 2.68 to 7 μmoles/g. Roughly the same proportion of polyethenoid fatty acids of both tissue and blood exists as fatty acid peroxides.

Summary. 1. Evidence is presented for presence of fatty acid peroxides in normal animal tissues and in blood. Total fatty acid fractions isolated from tissues yielded positive values with the thiobarbituric acid method and with indophenol dye and thiocyanate methods. Blood, which was not found to react positively by the 3 methods on isolated fatty acids, contained fatty acid peroxides from their ability to diminish induction period of the lipoxidase-linoleate system. 2. A modified method in presence of 1.2×10^{-4} M ferric chloride has been developed for thiobarbituric acid reaction, which apparently yields values for fatty acid peroxides in blood as well as in tissues.

We are indebted to Dr. Joseph L. Haining for suggesting that fatty acid peroxides could be demonstrated by their effect on induction period of lipoxidase-linoleate system.

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Utilization of Labeled Proteins in Synthesis of Tissue Proteins.* (24859)

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Numerous studies indicate that proteins administered parenterally can supply body protein needs over long periods of time(1-7). Howland and Hawkins(5) and Whipple and Madden(6) observed a positive nitrogen balance and no significant nitrogen loss in urine when plasma proteins were given to dogs intravenously, and increased excretion of nitrogen when plasma was given orally. These results were presented as evidence that injected plasma proteins are more completely utilized by the body than are orally administered proteins, and that injected plasma proteins can be utilized for synthesis of tissue proteins without prior breakdown to their constituent amino acids. Other workers(7-10), however, suggest that proteins administered intravenously diffuse into the lymphatics, and are then slowly metabolized, breaking down gradually and probably completely to amino acids. Yuile *et al.*(11) studied metabolism of C^{14}-lysine-labeled albumin and globulin administered intravenously to dogs. They found that transfer of radiocarbon from plasma to tissue proteins is accompanied by very small loss of radioactivity in urine and expired air; there was no evidence of complete breakdown to amino acids suggesting that plasma proteins are utilized in body economy as such, or after only partial catabolism within the cell. The following report presents data suggesting that isotopically-labeled proteins enter ascites cells *in vitro,* are utilized for synthesis of cell proteins, and do not appear to be completely broken down to their constituent amino acids.

Methods. Ehrlich mouse ascites carcinoma cells were obtained as described(12) and resuspended in 5 volumes of solution (Incubation Media) having following composition; 0.02 M potassium phosphate buffer, pH 7.8; 0.035 M potassium bicarbonate; 0.025 M potassium chloride; 0.004 M magnesium chloride; and 3 mg of heparin/100 ml. Two ml aliquots of the cell suspension were incubated with radioactive proteins or an equivalent amount of radioactivity as free amino acids for varying periods to 6 hours. At end of incubation the cells were recovered by centrifugation, washed twice with cold Incubation Media, precipitated with trichloroacetic acid and total acid-soluble and protein fractions isolated(13,14). In experiments in which the cellular fractions of ascites cells were prepared by differential centrifugation (15-17) following incubation, total acid-soluble and protein fractions were obtained from individual cellular fractions by similar procedure. Determinations of radioactivity were carried out by direct plating method(17). Ascites cell and plasma proteins were labeled *in vivo* by single intraperitoneal injections of 1 mg of DL-leucine-2-C^{14} (specific activity = 4.5 x 10^6 cpm/mg) or 4 mg S^{35}-L-methionine (specific activity = 6 x 10^6 cpm/mg), into tumor bearing mice. Forty-eight hours after injection of isotopic amino acid, the ascites tumor was removed and cells and plasma separated by centrifugation. Plasma proteins were dialyzed overnight against running water at 4°, then for 4 hours against 10 volumes of Incubation Media. An insoluble residue, formed during dialysis, was removed by centrifugation and discarded. The labeled ascites cells were lysed in distilled water(17), centrifuged at 144,000 x g for 2 hours, and the supernant dialyzed as described above.

Results. In vitro incorporation of radiocarbon from plasma proteins labeled with C^{14}-leucine or S^{35}-methionine, and from corresponding free amino acids is presented in Fig. 1. Approximately 16,000 cpm of C^{14}-leucine (1A) or C^{14}-plasma proteins (8 mg, 1B) and 40,000 cpm of S^{35}-methionine (1C) or S^{35}-plasma proteins (6 mg, 1D) were incubated with ascites cells for 1, 2, 4 and 6 hr in total volume of 3 ml. Transfer of C^{14} or S^{35} from plasma proteins to cell proteins occurred without accumulation of significant levels of acid-

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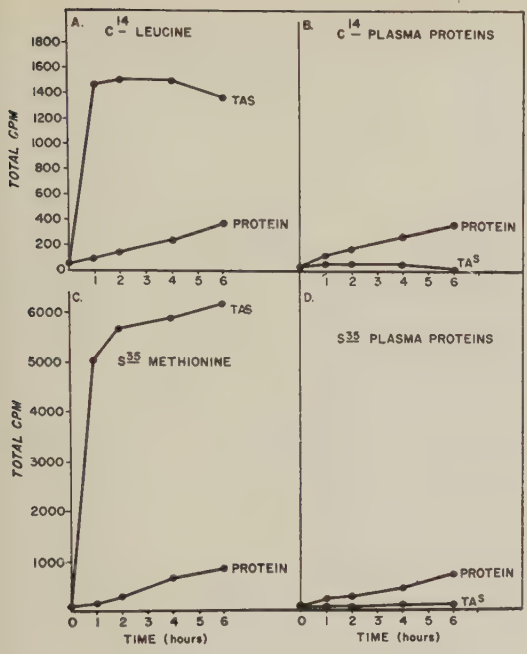

FIG. 1. Incorporation of radiocarbon into ascites cell protein and acid soluble fractions. Cells were incubated with C^{14}-leucine (A), C^{14}-labeled ascites plasma proteins (B), S^{35}-methionine (C) and S^{35}-labeled ascites plasma proteins as described in text.

soluble radioactivity whereas in experiments with free amino acids, markedly higher levels of acid-soluble radioactivity occurred concomitantly with incorporation into proteins.

Plasma proteins (140,000 cpm, 20 mg) and in a separate experiment cell proteins (180,000 cpm, 15 mg) previously labeled *in vivo* with S^{35}-methionine, were incubated with ascites cells for 1 hour in total volume of 5 ml. Following incubation, the cells were recovered by centrifugation, lysed in distilled water and separated into nuclear, mitochondrial, microsomal and supernatant fractions(17). A similar experiment was carried out with S^{35}-L-methionine (150,000 cpm). The results are summarized in Table I. Radioactivity was recovered in proteins of most fractions from cells incubated with labeled plasma proteins; no radioactivity was detected in acid-soluble fractions. Incubation with S^{35}-cell proteins obtained from the cellular supernatant of labeled ascites cells revealed significantly higher levels of radioactivity in proteins of cell fractions isolated, as compared to plasma protein incubations. Radioactivity was also detected in the acid-soluble fraction from nuclei, mitochondria and supernatant but not from microsomes; in most cases, however, protein-bound radioactivity was significantly greater than acid-soluble radioactivity. It should be pointed out that the nuclear fraction is probably contaminated to some extent with whole cells not lysed by this procedure.

Significant incorporation of radiosulfur from S^{35}-L-methionine into the proteins of various cellular fractions occurred during incubation period; non-protein radioactivity, however was higher in all fractions.

The small amounts of radioactivity found in acid-soluble fraction prepared from the incubating media after removal of ascites cells by centrifugation (Table I), may have been due to adsorbed free amino acids not removed by dialysis during preparation of labeled proteins or to incomplete precipitation of some labeled proteins used as substrates.

TABLE I. Incorporation of Radioactivity from S^{35}-Labeled Proteins and S^{35}-L-Methionine into Proteins of Ascites Cell Fractions.

	Total radioactivity					
	Plasma proteins		Cell proteins		L-methionine	
	t_o	1 hr	t_o	1 hr	t_o	1 hr
Total acid-soluble						
Nuclei	0	0	0	135	897	4,433
Mitochondria	0	0	0	291	0	125
Microsomes	0	0	0	0	0	1,320
Supernatant	0	0	162	548	4,218	34,080
Incubation media	3640	3948	3456	2183	146,000	106,000
Proteins						
Nuclei (8 mg)	78	350	104	4586	18	902
Mitochondria (1 ")	0	1	8	104	6	42
Microsomes (5 ")	26	41	19	2889	16	241
Supernatant (11 ")	198	1133	59	2931	5	1,078

Low levels of radioactivity recovered in acid-soluble fractions following incubation with labeled cell proteins suggest that these proteins may have been degraded to some extent to low-molecular weight products either as the result of catheptic hydrolysis or of metabolic degradation. Incubations with isotopic free amino acids led to accumulation of considerable amounts of free amino acids within the cell during incorporation into proteins; however, incubations with isotopic proteins led to labeling of proteins without significant accumulation of intracellular free amino acids. These results suggest that ascites cells can utilize proteins for synthesis of tissue proteins by pathways which do not involve complete breakdown to free amino acids or low molecular weight peptides. The observation that protein of cellular fractions of ascites cells contains radioactivity following incubation with labeled proteins, would indicate that the proteins used as substrates enter the cell and that protein labeling is not due to adsorption on cell surface as might be interpreted from studies with intact cells (Fig. 1). The failure of Fillerup *et al.*(18) to observe penetration and oxidation of C^{14}-labeled albumin by ascites cells may have been due to low levels of radioactivity used in their incubation.

It is interesting that incubations with soluble proteins obtained by high speed centrifugation of lysed radioactive ascites cells lead to a significantly greater labeling of cell proteins than with labeled ascitic plasma proteins.

Summary. Ehrlich ascites cells were incubated *in vitro* with radioactive amino acids or radioactive ascites proteins prepared biosynthetically. Incubations with free amino acids resulted in accumulation of large amounts of radioactivity in the acid-soluble fraction and in incorporation of radioactivity into the proteins. Incubations with labeled proteins resulted in labeling of cell proteins without significant accumulation of radioactivity in the acid-soluble fraction. Radioactive proteins were isolated from various cell fractions of ascites cells following incubation with labeled free amino acids or proteins. These results suggest that proteins enter ascites cells where they are utilized for synthesis of cellular proteins; free amino acids do not appear to be intermediates in this process.

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Galactose and Bile Flow.* (24860)

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Galactose and lactose have several physiologic properties not characteristic of other carbohydrates(1,2,3). Rats ingesting these sugars in sufficiently high amounts exhibit transient diarrhea, ruffed fur and paw edema and eventually develop cataracts. Galactosuria, polyuria and polydypsia occur. In some studies concerning dietary carbohydrates and gastrointestinal secretions another property peculiar to these sugars appeared. Bile volume flow is depressed following ingestion of these but not other sugars.

Method. Rochester colony albino rats (125 to 150 g) were maintained on diet of dog checkers. After 24-hour fast they were anaesthetized with di-ethyl ether and the bile duct cannulated with polyethylene tubing. In some animals the carotid artery was similarly cannulated. Animals were then placed in restraining cages and allowed to recover from the anesthesia. Bile flow was measured in 30-minute periods by means of graduated pipette attached to polyethylene cannula. After 4 or more determinations of flow during fasting state, the animals were administered a test dose of carbohydrate by stomach tube or *via* intra-arterial cannula. Volume flow determinations were continued for 6 or more periods. Collected bile was not returned to animal. Calcium analyses were done by flame photometer.

Results. During fasting control periods, flow rates in individual rats varied less than 5% but the range was 0.25 ml of 0.80 ml/hour. In each animal average flow rates during 4 or more fasting periods were averaged and this flow rate assigned a value of 100%. Flow rate during each period was computed as percentage of average control rate of flow.

Every sugar tested by intra-arterial injection caused a transient drop in flow lasting 30 minutes. In Table I are summarized data on flow rates as percentage of control during a 2 hour interval, starting 30 minutes after administration of test sugar. Glucose administered by stomach tube or by intra-arterial injection did not modify bile volume flow. Of several pentoses tried a small and barely significant depression in flow followed only administration of L-xylose and D-lyxose. Preliminary observations suggest that fructose and sucrose likewise do not change bile flow. Galactose in dosages of one mg and more/g of body weight causes depression in bile flow lasting 2 or more hours. Fig. 1 shows time course of bile flow following oral administration of several dosages of galactose. In general response is proportional to dosage used. Dosages of 25 mg of this sugar given intra-arterially appear to enhance slightly volume flow. Lactose administered orally depressed bile flow to lesser extent than would be predicted on basis of its galactose content. This may be related to differences in absorptive rates of the monosaccharide administered alone as compared to galactose in the disaccharide which must first be hydrolyzed.

When protein was fed along with galactose or lactose the depression in bile flow was much less marked. In animals administered 500 mg of lactose plus 200 mg of protein (egg albumin) volume flow averaged 103 ± 6% of control flow. Following feeding of 500

TABLE I. Volume Flow of Bile.

Sugar	Dosage	Route	No. animals	Bile flow (% control flow)
Glucose	500	Oral	9	100 ± 7†
	250	I.A.*	9	96 ± 4
Galactose	250	Oral	9	75 ± 4
	500	"	8	59 ± 5
	25	I.A.	9	114 ± 4
	250	"	10	65 ± 5
Galactose + glucose	250 each	Oral	9	73 ± 6
Lactose	500	"	9	82 ± 4
D-ribose	200	I.A.	4	107 ± 4
D-arabinose	"	"	7	100 ±11
L-arabinose	"	"	7	106 ± 6
D-lyxose	"	"	4	88 ± 4
L-xylose	"	"	6	86 ± 4

* Intra-arterial. † Mean ± stand. error.

* Supported by research grant from Nat. Inst. Health.

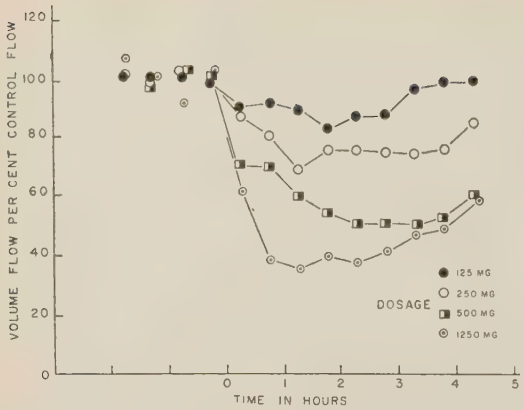

FIG. 1. Bile volume following oral galactose administration.

mg of galactose plus 300 mg of protein, volume flow of bile averaged 81 ± 2% of control rate in comparison to 59% when this quantity of galactose was given alone.

When volume flow remained constant there was no change in total solids content of bile collected during 2 successive periods before and after feeding. In 8 animals, at flow rate of 0.35 ml/hour total solids were 3.4 ± 0.4% in both periods. Inorganic solids were 1.18 ± 0.11% during first period and 1.08 ± 0.08 during second period. In 8 animals in which flow rates decreased from average of 0.50 ± .02 to 0.35 ± .02 ml/hour due to galactose administration, solids content likewise remained unchanged. In fasting period total solids were 3.60 ± .20% with 1.13 ± .07% inorganic solids. In the period following galactose administration with lower flow rates, total solids were 3.50 ± 0.37% and inorganic solids were 1.08 ± .06%. Calcium concentration in bile was likewise unchanged when volume flow was reduced by galactose administration. In 15 animals at flow rates of 0.48 ml/hour calcium concentration was 6.57 ± .42 meq/liter as compared with 5.98 ± .54 meq/liter when flow rate reduced to average of 0.29 m/hr.

The mechanism by which depression of bile flow is produced is unknown. Galactose has a trans arrangement of the hydroxyl groups on carbon atoms 4 and 5. Glucose, fructose and 3 of pentoses used that did not influence bile flow have a cis arrangement of these comparable hydroxyl groups. The 2 pentoses that produced small reductions in bile volume flow have the same arrangement of these hydroxyl groups as does galactose. This raises the possibility of stearic induced inhibition of secretion of bile. Further studies are planned using other carbohydrates with cis and trans arrangements of these hydroxyl groups and the effect of increasing amounts of glucose on inhibition of bile flow by standard dosage of galactose.

Summary. Galactose and lactose depressed volume flow of bile in the rat. This depression is in general proportional to dosage of galactose administered. Addition of protein to test meal of galactose or lactose lessens the effect of these sugars on bile flow. Since composition of bile as to inorganic and organic solids and calcium remained constant at different rates of flow, the excretion of these substances is proportional to volume flow.

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In vitro Inhibition of Growth of *M. tuberculosis* by Certain 11-Oxygenated Steroids. (24861)

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It has been demonstrated in numerous animal experiments that glucocorticoids decrease the inflammatory response of host to tuberculosis(1). There is ample clinical evidence that administration of these hormones in absence of adequate antibiotic coverage may activate latent tuberculosis in man(2). Despite this large body of evidence about *in vivo* effects of 11-oxygenated steroids, little is known about the effect of these hormones on the tubercle bacillus itself. We found that certain of these hormones can exert a profound suppressant effect on mycobacterial growth *in vitro*.

Procedure. A standard inoculum, 0.1 cc of a 1 x 10^{-3} dilution of stock cultures in logarithmic growth phase at O.D. .509 of H 37 RV and several local isoniazid sensitive and resistant strains of *M. tuberculosis* was used in all studies. Crystalline hydrocortisone or corticosterone was added in several concentrations in distilled water to the following synthetic culture medium: (Aldridge, Muchmore and Felton—in preparation)

Potassium phosphate, monobasic	5.	g
Ammonium chloride	5.	g
Magnesium citrate	1.	g
Potassium sulfate, dibasic	.5	g
Tween 80, certified	.2	ml
Ferric ammonium citrate	.0025	g
Distilled water, to make	980.	ml

Adjust to pH 6.8 with solid KOH; autoclave; add 20 ml of sterile 50% glucose. Bacterial growth was determined by measuring optical density at 525 mμ at various intervals after inoculation. Optical density values represent a mean of 4-5 culture tubes in each instance.

Results. In 5 experiments with 3 isoniazid sensitive strains 100 μg/ml of hydrocortisone produced a striking decrease in bacterial growth and marked increase in apparent lag phase. None of the bacilli grown in this concentration of hydrocortisone entered the usual accelerated growth phase, although some escape from steroid effect was sometimes seen at 22-24 days after inoculation. Ten μg/ml produced slight slowing of growth in some strains. One μg/ml was without apparent effect. Isoniazid resistant organisms isolated from same patients following unsuccessful isoniazid treatment were similarly inhibited by hydrocortisone.

Growth of standard strain H 37 RV obtained from Trudeau Laboratory, was also inhibited by hydrocortisone at concentration of 100 μg/ml. Twenty μg/ml produced slight slowing of growth (Fig. 1). In 1 sensitive and 1 resistant strain 100 μg/ml of corticosterone produced similar depressant effects. In both these strains 1 and 10 μg/ml produced slight slowing of growth. Table I summarizes these data.

No change in acid fastness of bacilli grown in these various concentrations of steroid was noted.

Hydrocortisone inhibition of bacterial growth is apparently limited to certain species. In 8 experiments no effect of hydrocortisone in concentrations of 100 μg/ml could be detected on growth or generation time of *E. coli* isolated from blood of patient who showed

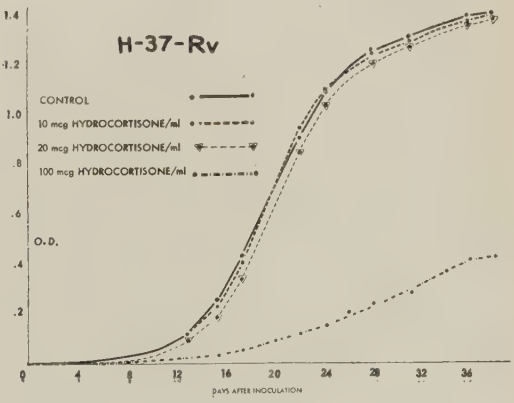

FIG. 1.

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† Oklahoma Tuberculosis Assn. Trainee.

TABLE I. Per Cent of Control Optic Density at Approximate Mid-point of Growth Curve.

	INH-S					INH-R			
	L-21-S	L-22-S (1)	L-22-S (2)	L-22-S (3)	H-37-RV	L-21-R	L-22-R (1)	L-22-R (2)	L-22-R (3)
Control	100	100	100	100	100	100	100	100	100
Hydrocortisone									
1 μg/ml	93	93	99			96	102	98	
10	76	89		87	100	75	92	95	98
20				81	87				78
100	23	22	36	29	13	34	21	29	23
Corticosterone									
1 μg/ml		86					82		
10		67					71		
100		10					19		

dramatic improvement after I.V. hydrocortisone semi-succinate.

Discussion. 11-oxygenated adrenal steroids have been previously shown to depress multiplication of cells. Glucocorticoids profoundly suppress mitoses in rat epidermis in culture with glucose, fructose, lactate, or pyruvate as substrate(3). This effect has been confirmed *in vivo*(4).

Perhaps more closely related to the present study is the work of Lester, Stone, and Hechter(5) with *Neurospora crassa*. These authors have recently shown that desoxycorticosterone at concentration of 250 μg/ml of culture medium produces approximately 50% suppression of mycelial growth. Hydrocortisone and cortisone were inactive at comparable concentrations(5). In another study Lester and Hechter found that desoxycorticosterone can inhibit growth of many Gram positive bacteria, but is ineffective against Gram negative bacteria except for *N. catarrhalis*(6). Hirsch, on the other hand, has found that cholesterol stimulates growth of *M. tuberculosis* at a concentration of approximately 100 μg/ml(7). Cortisone had no effect on growth of *M. tuberculosis in vitro*. However, when tubercle bacilli were placed in collodion coated bags into the peritoneal cavity of guinea pigs, parenteral administration of cortisone decreased multiplication of the bacilli(8).

Concentration of corticosteroid which markedly inhibits *in vitro* mycobacterial growth is well above that obtainable in blood after rapid I.V. injection of 100 mg(9). However, corticosteroids may be concentrated in tissues (10). Further, under certain circumstances such as intrathecal administration of hydrocortisone for tuberculosis(11) concentrations in spinal fluid may equal or exceed those required for marked inhibition *in vitro*.

It is possible that the total effect of administration of certain corticosteroids may be a balance between an effect on decreasing multiplication of *M. tuberculosis* and an effect on decreasing host response to infection, the latter predominating under usual circumstances. There is, however, no necessary correlation between depression of host response and suppression of bacillary growth. Corticosterone, though possessing only 1/3 - 1/5 the anti-inflammatory potency of hydrocortisone in man, is as effective as hydrocortisone in inhibiting mycobacterial growth.

A bacterial system of this type may perhaps be of use both for determining nutritional requirements of the tubercle bacillus and for studying *in vitro* effects of certain mammalian steroid hormones.

Summary. Hydrocortisone and corticosterone in a concentration of 100 μg/ml markedly inhibit growth of isoniazid sensitive and resistant strains of tubercle bacilli grown in a simple synthetic culture medium. Ten and 20 μg/ml produce slight slowing of growth in some strains.

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Effect of Certain Androgenic Steroids and Cortisone on Gastric Ulcerogenesis in Fasting Rats. (24862)

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When intensive corticoid treatment is associated with 4 days fasting in rats, steroid gastric ulcers develop in about 90% of animals. These ulcers are always found in glandular portion of stomach and closely resemble human peptic ulcers. Fasting alone, on the other hand, results in forestomach ulcers, similar to typical lesions of the Shay rat. Robert and Nezamis(1) propose this technic of ulcer production as assay for ulcerogenic property of steroids. Several factors may be responsible for steroid gastric lesions. Most frequently quoted hypotheses concern post-steroid changes in gastric secretion, antiphlogistic role of corticoids and possible decrease of gastric mucus synthesis(1,2). We showed that sulphated mucosubstances are reduced in both gastric tissue and gastric juices of rats treated with cortisone(3). The mechanism of this steroid action is, however, not clear. That corticoids exert a general anti-anabolic effect will be taken into consideration when interpreting any local effect of these hormones. It is known, for example, that the effect of cortisone on tissue healing and on synthesis of collagen is anti-anabolic and that the antiphlogistic role of corticoids is partly explained by this effect(4,5). Some anti-anabolic effects of cortisone may be offset however by certain anabolic androgens(5,6,7), and it seemed interesting to investigate this problem using above mentioned method of producing cortisone ulcers in rats. The present report concerns investigation of possible protective action of some synthetic androgens on development of gastric ulcers in fasting cortisone treated rats.

Materials and methods. Male rats of Wistar strain, of body weight 195 to 220 g were placed in individual cages and fasted 4 days. Some animals received hormonal treatment for 10 days preceding fasting period. 17-ethyl-19-nortestosterone (E.N.T.) (Nilevar, Searle & Co.) was given 5 mg/os daily, crushed tablet mixed with food(5). Testosterone enanthate (T.E.) (Delatestryl, Squibb & Sons) was injected intramuscularly, one 100 mg dose 10 days before beginning of fasting. Cortisone acetate (Merck & Co.) was injected subcutaneously in 10 mg dose once daily, only during fasting period(1). The following 6 groups of rats were used: 1. Pretreated with E.N.T. before fasting; 2. Pretreated with T.E. before fasting; 3. Fasted only; 4. Pretreated with E.N.T. and given cortisone during fasting; 5. Pretreated with T.E. and given cortisone during fasting; 6. Given cortisone during fasting. Isotope was administered 24 hours before killing of rats. Radiosulphur (S^{35} in H_2SO_4, Atomic Energy, Canada) was injected subcutaneously, one mc/kg body weight. The dose was dissolved in 5 ml distilled water together with 40 mg of sodium sulphate(6). Animals were killed with ether, the stomachs dissected, opened along great curvature and the mucosa examined under dissecting microscope (X 10). Stomachs were then studied for S^{35} radioactive mucopolysaccharides following modification(3) of method described previously(8,9).

Livers were dissected for total cholesterol determination(10) and gastrocnemius muscle was removed for study of skeletal muscle electrolytes. Baird Flame Photometer was used for determination of sodium and potassium extracted from the tissue(11). Chlorides were determined by titration(12).

Results. Body weight loss during 4 days fasting period averaged 28, 29, 27, 32, 28 and 33% in 6 groups of rats respectively.

Mortality rate was not affected by E.N.T. but markedly increased in group of rats pretreated with T.E. Cortisone associated with fasting promoted surviving rate of animals whether or not pretreated with E.N.T. and T.E. (Fig. 1). Only rats which survived complete fasting period were examined for gastric lesions.

In rats receiving any type of steroid treatment, ulcers were found only in glandular portion of stomach. Control fasting rats (Group 3) had ulcers mostly in forestomach, sometimes associated with ulcers of glandular part. Macroscopic appearance of these lesions agreed with the description of Robert and Nezamis(1) Fig. 1 shows percent of animals which developed gastric ulcers and where these lesions were localized. Occurrence of ulcers in glandular stomachs was increased in T.E. pretreated rats, fasted only,

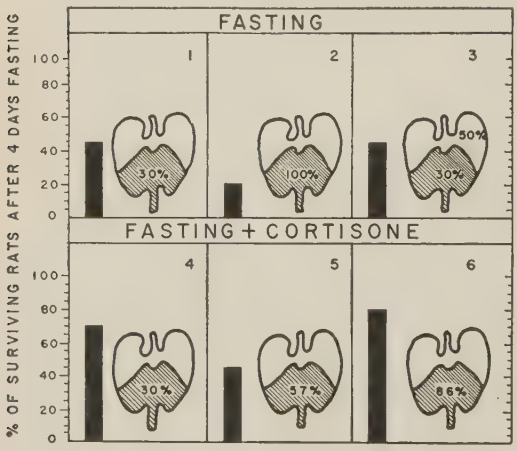

FIG. 1. Percentage of fasting rats surviving (black columns) and of rats with ulcers of glandular stomach (crossed area) and forestomach (open area). Rats of groups 1 and 4 treated with E.N.T. (17-ethyl-19-nortestosterone) and rats of groups 2 and 5 treated with T.E. (testosterone enanthate) prior to fasting and cortisone.

TABLE I. Total Radioactivity of Gastric Tissue in Fasting Rats (cpm/g of Wet Tissue) and % of S^{35} Taken up by Gastric Fraction Containing Sulphated Mucopolysaccharides (S.M.). Mean and range.

Group	Treatment*	No. of rats	cpm/g w.t.	S.M., %
3	None	13	414 (179–552)	73.4 (58.8–86.5)
4	E.N.T. & cortisone	18	314 (197–509)	65.1 (58.0–70.5)
5	T.E. & cortisone	12	267 (179–320)	59.1 (47.9–60.1)
6	Cortisone alone	23	229 (103–320)	44.5 (29.1–49.2)

* Treatment with E.N.T. (17-ethyl-19-nortestosterone) and T.E. (testosterone enanthate) preceded fasting and cortisone.

but this steroid plays some protective action in cortisone treated fasted animals. E.N.T. does not influence occurrence of ulcers in glandular stomachs of fasting rats; it has however very marked protective action in animals treated with cortisone. Cortisone alone results in high incidence of ulcers in glandular stomachs.

Table I presents data on total radioactivity and percentage of S^{35} in fraction considered to contain gastric sulphated mucopolysaccharides (S.M.). This study was performed only in rats of Groups 3, 4, 5 and 6. Rats of Group 3, fasted only, served as controls for fasting animals treated with cortisone alone (Group 6) or pretreated with androgens prior to cortisone and fasting (Groups 4 and 5). Radioactivity of fraction S.M. is reduced in all fasting cortisone treated rats as compared with rats which were fasting only, or which received E.N.T. prior to cortisone and fasting. Pretreatment with T.E. was less effective.

Table II summarizes data on electrolyte content of skeletal muscle and on liver cholesterol.

Discussion. Synthetic steroid 17-ethyl-19-nortestosterone (E.N.T.) is considered a highly anabolic hormone with a low androgenic effect(13). Its protective action against the anti-anabolic effect of cortisone on connective tissue seems to be well demonstrated (5,6,7). Testosterone enanthate (T.E.), on the other hand, is considered a highly androgenic steroid(14). Prolonged duration of ac-

TABLE II. Electrolytes of Skeletal Muscle and Total Cholesterol of Liver in Fasting Rats. Mean and range. 6 groups.

Treatment*	No. of rats	Muscle Cl (meq/100 g fat-free solids)	Muscle Na (meq/100 g fat-free solids)	Muscle K (meq/100 g fat-free solids)	Liver total cholesterol (mg/100 g wet tissue)
E.N.T.	12	10.3 (10.3–11.9)	12.0 (9.5–14.0)	40.3 (36.1–42.8)	316 (226–384)
T.E.	8	10.5 (10.0–11.1)	12.2 (9.6–13.1)	39.1 (35.4–40.5)	253 (258–316)
None	13	10.2 (9.9–10.6)	11.0 (10.0–12.1)	40.7 (37.7–42.2)	284 (222–395)
E.N.T. and cortisone	18	11.0 (8.5–12.3)	11.1 (8.8–12.8)	43.4 (40.3–46.1)	307 (218–450)
T.E. and cortisone	12	9.8 (8.5–11.6)	11.5 (9.3–13.4)	41.4 (38.5–44.2)	311 (216–404)
Cortisone	23	9.7 (9.1–11.8)	10.9 (9.2–12.7)	42.8 (37.0–46.4)	262 (116–337)

* Treatment with E.N.T. (17-ethyl-19-nortestosterone) and T.E. (testosterone enanthate) preceded fasting and cortisone.

tion of an injection makes the use of T.E. especially practical.

E.N.T. and T.E. represent predominantly anabolic and predominantly androgenic steroids respectively. They were tested for ulcerogenic property in fasting rats treated or not treated with cortisone. Because of possible effect of steroid hormones on gastric mucus production, the content of S^{35} sulphated mucosubstances in stomachs of some rats was also studied. As lipid and electrolyte metabolism is knowingly influenced by both fasting and steroid treatment, some supplementary investigations on this subject were also performed.

The high mortality rate in this experiment, as the result of fasting alone or fasting combined with steroids, presents a serious complication, not mentioned by authors proposing steroid ulcerogenesis as assay procedure(1).

It is apparent that cortisone added to fasting, increases survival of rats and that E.N.T. and T.E. have not such a protective action. Steroid treatment does not protect against loss of body weight due to fasting. It is further apparent that pretreatment of rats with E.N.T. gives very marked protection against ulcers in cortisone treated rats. This effect is comparable to that observed in our previous work on E.N.T.(5,6,7,15).

Administration of cortisone during fasting period interferes with uptake of S^{35} by the fraction of gastric tissue, considered to contain sulphated mucopolysaccharides (S.M.). This agrees with previous work on effects of cortisone on metabolism of certain mucopolysaccharides(16) and with our data on post-cortisone utilization of S^{35} by rats' stomachs (3). This significant decrease of S^{35} uptake by S.M. fraction is offset in animals which received E.N.T. prior to cortisone treatment. It is apparent that the anabolic steroid E.N.T. has a somewhat opposite action on S^{35} uptake than cortisone(5,6,7). A possible parallelism between occurrence of ulcers and S^{35} uptake by S.M. fraction of gastric tissue might be considered. This hypothesis, however, needs more precise and detailed study on larger number of animals.

Neither electrolytes of skeletal muscle nor liver cholesterol were significantly affected by steroid treatment or fasting. This may be due to the short duration of experiment.

Summary. The effect of 17-ethyl-19-nortestosterone (E.N.T.) and testosterone enanthate (T.E.) on development of gastric ulcers in fasting cortisone treated rats was studied. Pretreatment of rats with E.N.T. gave a very marked protection against ulcers in cortisone treated rats. It was also noted that S^{35} uptake by sulphated mucopolysaccharides of gastric tissue was significantly reduced in cortisone treated fasting rats as compared with rats fasting only, or those given E.N.T. prior to cortisone. Androgen T.E. had no marked protective action. No

direct parallelism was noted between surviving rate and ulcer occurrence in steroid treated fasting rats.

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Influence of Hypothermia on Secretory Activity of Rabbits' Appendix and on Closed Duodenal Loops.* (24863)

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Experimental observations previously reported demonstrated that rupture of obstructed appendix in rabbits, chimpanzees and man was caused by early development of high intraluminal pressures resulting from rapid rate of appendical secretion(1,2). In a large series of observations upon other species in which activity of appendix was examined, only in chimpanzee and man was there evidence of active secretion. Morton and Sullivan(3) compared closed jejunal loops with similar ones in the ileum. The intra-enteric pressure of jejunal loops (52 cm of water) was 7 times that developed by ileal (8 cm) segments. They concluded that the jejunum secreted more rapidly than the ileum. No observations of duodenal secretory activity were made. Recent observations(4) from this laboratory showed that local gastric cooling in a variety of species, including man, reduced gastric secretory activity and proteolytic digestion. This suggested that the influence of hypothermia upon secretory activity of the obstructed appendix and of closed duodenal loops should be assessed.

Methods. Adult white rabbits weighing 1.5 to 2.0 kg were anesthetized and opened by midline abdominal incision. Appendical closed loops were established by ligating base of appendix with umbilical tape. Closed duodenal loops about 8 inches long were made by occluding the lumen with ligatures at 2 sites to exclude biliary and pancreatic secretions. Integrity of the vascular arcades of these segments was carefully preserved. Rate of appendical secretion was investigated using the simple closed loop described above and in a modified preparation. In the latter, so that secretions could be collected with minimal change in intraluminal pressure, the appendix was cannulated with a plastic tube to which a condom was attached. Duodenal secretions were collected only from unmodified closed loops. In those rabbits that were used for in-

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TABLE I. Volume of Secretion from Appendix and Duodenal Closed Loops in 6 Hours. (6 rabbits used in each temperature range.)

	Systemic temp. °C			Local temp. °C	
	38	27–30	21–24	38	15
	ml				
Appendix					
Without drainage					
Mean	10.5	3.8		16.0	6.6
Range	9–12*	2–7		14–18*	3–11
With drainage					
Mean	17.2	3.7		18.3	4.5
Range	11–24.5	2–6		15–22	4–5
Duodenum					
Without drainage					
Mean	36.5		3.5	44.5	4.3
Range	35–38		3–4	32–51†	4–5

* 3 perforations.
† 2 " .

traduodenal and intra-appendical pressure determinations, a #280 polyethylene catheter 2.5 mm in diameter was inserted into the lumen, the remainder of tube raised vertically to act as pressure manometer. In all animals undergoing systemic hypothermia as well as in normothermic controls, the obstructed segment of bowel was returned to abdominal cavity and the incision closed. In animals in which appendixes or closed duodenal loops were to be cooled locally, the portion of bowel to be studied was kept outside the abdominal cavity. Controls were employed for this latter group by exteriorizing the appendix or closed duodenal loop and warming it externally to 38°C. General body hypothermia was achieved by briefly immersing animals in ice water. Local hypothermia or normothermia of obstructed loops was provided by circulating cool or warm (38°C) solution of desired temperature through plastic bags in close approximation to exteriorized segment. The bags were arranged in such a way that they exerted only minimal pressure on obstructed loop. All studies, both secretory and manometric, were uniformly 6 hours long.

The results of experiments with rabbit appendix are shown in Table I. These observations indicate that both local and systemic hypothermia markedly reduced rate of secretion. Volumes secreted by obstructed appendixes, whether drained or closed loops, during systemic hypothermia were essentially the same. Normothermic appendixes secreted 3 to 5 times as much as cooled appendixes during similar time periods. A reduction in secretory volumes of equal magnitude was attained in drained appendixes cooled locally.

Among 12 rabbits of control group there were 6 perforations attending obstruction of appendix, an incidence of 50%. Appendical pressure studies (Table II) parallel the finding of decreased volumetric secretion in hypothermic appendixes. Pressures in group undergoing systemic hypothermia, after 6 hours of obstruction, are uniformly lower than those in normothermic control group. Mean pressure in former is approximately one-fifth that recorded in latter group. Similar observations were made in comparing secretory rates of appendixes where temperature was controlled locally.

Perforation of the appendix occurred in 8 of 12 normothermic rabbits of control groups for pressure experiments, an incidence of 66%. Five of 8 occurred in appendixes locally warmed, (38°C) outside abdomen, while the remaining 3 were observed in animals at normothermic systemic temperatures (38°C). Perforation was not observed in cooled appendixes.

Data in Table I demonstrate that volume of duodenal secretion is reduced markedly by systemic and local hypothermia. In fact, volumes secreted during either form of hypothermia are only about one-tenth as large as those secreted under normothermic (38°C) conditions. Spontaneous duodenal perforation at sites away from ligatures and within limits of experimental period, occurred in 2

TABLE II. Pressure Developed in Appendix and Duodenal Closed Loops in 6 Hours. (6 rabbits used in each temperature range.)

	Systemic temp. °C			Local temp. °C		
	38	27–30	22–27	38	20	15
	cm					
Appendix						
Mean	67.5	13.2		82.8		8.0
Range	42–84*	7–21		72–98†		3–15
Duodenum						
Mean	70.5		8.4	61.9	11.5	
Range	53–87		5–13	42–75‡	7–18	

* 3 perforations.
† 5 " .
‡ 1 " .

of the 6 exteriorized duodenums locally warmed. There were no perforations in normothermic obstructed duodenal loops that remained within the abdominal cavity, or in any of those kept hypothermic, whether in or out of the abdomen. The data in Table II show that both systemic and local hypothermia virtually stopped the rise of pressure in duodenal closed loops. Perforation of the duodenum occurred in one animal undergoing local warming when the intraluminal pressure reached 75 cm of fluid.

Discussion. Our earlier studies(5) showed that maintenance of an intraluminal pressure of 40 cm of water caused portions of a jejunal closed loop to become nonviable within 20 hours. Nonviability and perforation of obstructed appendix occur in a much shorter time because of rapidity of elevation and the magnitude of intraluminal pressures (up to 136 cm of water). Pressures such as these cause perforation through agencies of venous stasis, ischemia and finally necrosis of areas of bowel wall. The duodenal closed loop also has a strong secretory capacity, being able to achieve high intraluminal pressures within a few hours. Some areas of duodenal wall in these segments, appeared somewhat dark and ischemic at end of 6 hours; one externalized, locally warmed and 2 intra abdominal normothermic duodenums perforated spontaneously. The low incidence of perforations in this group, when compared with the great frequency of perforation in the obstructed appendix is attributable, undoubtedly, to lower intraluminal pressure ranges developed in closed duodenal loops. Blood pressure carefully monitored during cooling, in a number of animals undergoing either appendical or closed duodenal loop obstruction, showed that cooling caused blood pressure falls of only about 5 mm of Hg, when recorded at temperatures between 28° to 31°C. Depression of blood pressure was therefore not responsible for the observed reduction in rate of secretion in locally cooled appendixes in otherwise normothermic rabbits. Studies of blood flow in locally cooled appendix or duodenum have not been made. However, we observed that local gastric cooling to 15°C in the dog is attended by mean reduction in gastric blood flow of 66% (unpublished). Undoubtedly, a similar occurrence attends local cooling in other portions of bowel. Diminished blood flow and decreased cellular activity of actively secreting epithelial cells are probably the agencies essentially responsible for the observed decrease in secretion and intraluminal pressure in closed segments of duodenum and in the obstructed appendix.

Conclusions. (1) In rabbits, hypothermia reduces rate of secretion in duodenal and appendical closed loops. Depression of secretion is reflected in failure of these loops to attain high intraluminal pressures found in normothermic control animals. (2) Local and systemic hypothermia prevented perforation of obstructed appendix or closed duodenal loop in the rabbit.

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Connective Tissue: I. Age and Sex Influence on Protein Composition of Rat Tissues. (24864)

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The aging process as currently understood is intimately connected with formation of new collagen(1,2,3,4), and modification of existing elastic tissue(5,6,7,8,9). However, the studies have involved principally aorta and skin; little is known about above processes of other tissues. In previous work on connective tissue obtained by implantation of polyvinyl sponges (Ivalon)*, collagen content/g of sponge implanted, increased with age of tissue(10). Collagen formed rapidly during first 2 or 3 weeks after implantation of sponge and then much more slowly. The present study was undertaken to determine in various tissues of rat, the relation of age and sex to content of soluble protein, soluble collagen, insoluble collagen and elastin. Such data would show whether tissues other than aorta and skin exhibit comparable changes with age.

Methods. Male and female rats, Wistar strain, of 3-5 weeks, 8 months and 2 years of age were used. Samples (0.1-1.0 g) of tail tendon, aorta, skin, uterus, lung, muscle, heart, liver, kidney and spleen were homogenized in 10 ml saline in Virtis "23" homogenizer for 5 minutes. Each homogenate was centrifuged at 20,000 x g for ½ hr and supernatant (saline soluble protein) was decanted. The residue was suspended with occasional stirring, in 20 ml 0.1 N NaOH and remained at 25° over night. The resulting mixture was centrifuged at 30,000 x g for ½ hr and the supernatant removed. The residue was extracted a second time with 10 ml 0.1 N NaOH for 2 hr at 25° and centrifuged as above. The 2 extracts, which contained the alkali-soluble protein (containing soluble collagen) were then combined. The above saline soluble and alkali-soluble protein fractions were together designated "soluble protein" (Fraction I). The residue was treated with 10 ml 0.1 N NaOH at 100° for 15 min, the mixture centrifuged at 20,000 x g for ½ hr and supernatant removed. This extraction was repeated once for all tissues and twice for skin. The hot alkali extracts of all samples were combined and designated as "insoluble collagen" (Fraction II); the residue from these extractions contained "elastin" (Fraction III). All protein extracts were concentrated to 5 ml and then hydrolyzed in 6 N HCl in sealed tubes at 110° for 16 hr. The hydrolysates were filtered and made to convenient volume. Nitrogen was determined by Conway microdiffusion method(11) and protein calculated as 6.25 x nitrogen. Hydroxyproline in the soluble protein fraction was determined by the method of Neuman and Logan(12). Soluble collagen was calculated by multiplying amount of hydroxyproline by 7.46(13). Total collagen represented the sum of soluble and insoluble collagen values. By "total noncollagen protein" is meant the difference between soluble protein and soluble collagen. Detailed data for male rats has been omitted from Tables except where significant sex differences were observed. All of these have been combined and recorded in Table VI.

Results. Soluble protein (Tables I and VI). Values for soluble protein were constant and independent of age and sex in the following tissues: voluntary muscle, kidney, lung, aorta, liver and spleen. For both sexes, a decrease in soluble protein with age was observed in tendinous, cardiac and dermal structures. Similar decreases were seen in uterine musculature. At 3-5 wks of age, the tail tendon of male contained more soluble protein than that of female rat. More soluble protein was found in connective tissue of skin of 8 mo female rats than in that of males (Table VI).

Insoluble collagen (Table II). Neither age nor sex appeared to have a consistent influence upon insoluble collagen content of connective tissue of upper leg muscle, kidney,

* Ivalon Surgical Sponge, Clay Adams Corp., N. Y.

TABLE I. Influence of Age on Content of Soluble Protein (g of Soluble Protein/100 g of Tissue) in Female Rat.

Tissue	Age Group	3-5 wk A	8 mo B	2 yr C
Upper leg muscle		17.6 ± 1.1 (4)	19.6 ± 1.6 (7)	19.4 ± 2.3 (3)
Lower " "		20.7 ± 2.4 "	19.9 ± 1.4 "	17.2 ± 1.6 "
Abdominal "		18.6 ± 3.6 (3)	17.9 ± 1.0 "	20.0 ± 1.1 (2)
Kidney		18.6 ± 2.3 (4)	15.8 ± 2.2 "	15.7 ± .5 (3)
Lung		17.3 ± 1.8 (3)	15.5 ± 1.7 "	16.1 ± .7 "
Liver		17.7 ± 3.4 (4)	20.7 ± 1.0 "	18.5 ± .5 "
Spleen		19.1 ± 2.0 (3)	19.3 ± .9 "	18.3 ± .8 "
Tendon		18.5 ± 1.2 "	16.7 ± 5.8 (4)	*6.5 ± 1.8 (4)*
Heart		19.0 ± 1.1 "	18.7 ± .9 "	*14.8 ± .3 (3)*
Aorta		7.7 ± 1.0 (4)*	7.3 ± 1.1 (6)	7.2 ± .8 (4)
Skin		13.5 ± 1.2 (3)	13.4 ± 2.0 (4)	*9.1 ± .9 (4)*
Uterus		14.5 ± 1.5 "	9.3 ± 2.5 (3)	11.6 ± 3.1 "

Values represent means with stand. dev. immediately following.

Figures in parentheses represent No. of animals and corresponding determinations unless otherwise specified.

Figures showing significant differences within the 5% level of confidence are italicized. Data in male rats are comparable to those in female, except as shown in Table VI, where only significant differences are recorded.

* Each determination consists of pooled sample of 5-6 aortas.

lung, liver or spleen. In both sexes, there was an increase with age in insoluble collagen of connective tissue of lower leg and abdominal muscles, tendon, aorta and skin. In the uterus a similar change occurred, which reached a plateau at 8 mo. In cardiac muscle insoluble collagen decreased with age. However, this decrement reached its maximum at 8 mo of age and remains unaltered at 2 yrs of age. The highest values for insoluble collagen at all ages were observed in the skin; at 3-5 wk, the descending order of magnitude in other tissues was as follows: tendon, aorta, uterus, abdominal muscle, leg muscle, heart, lung, spleen, liver and kidney.

More insoluble collagen was present in the abdominal muscle of females at 3-5 wks and at 8 mo than in males of corresponding ages. The insoluble collagen of tendon was higher at 3-5 wks in females than in males, but lower in females than in males at 8 mo. At 8 mo of age, more insoluble collagen was present in the male than in female aorta; at other ages studied no significant sex differences were observed. A significantly greater amount of insoluble collagen was present in skin of female as compared with male at 3-5 wk of age, while the reverse was true at 8 mo of age (Table VI).

Elastin (Table III). In relation to age

TABLE II. Influence of Age on Content of Insoluble Collagen (g of Insoluble Collagen/100 g of Tissue) in Female Rat.

Tissue	Age Group	3-5 wk A	8 mo B	2 yr C
Upper leg muscle		1.0 ± .5 (4)	1.2 ± .3 (3)	1.0 ± .4 (3)
Lower " "		1.0 ± .8 "	1.4 ± .3 (4)	1.8 ± .8 "
Abdominal "		1.5 ± .2 (3)	1.7 ± .4 "	*2.2 ± .4* "
Kidney		.2 ± .1 (4)	.5 ± .1 (7)	.4 ± .1 "
Lung		.9 ± .2 "	1.4 ± .2 (5)	1.0 ± .1 "
Liver		.2 ± .0 (3)	.2 ± .1 (7)	.1 ± .0 "
Spleen		.8 ± .2 (4)	.5 ± .1 "	.6 ± .3 "
Tendon		4.5 ± .5 (3)	*10.3 ± 1.5 (4)*	*15.7 ± 2.0 (4)*
Heart		.9 ± .2 "	*.6 ± .1* "	.7 ± .2 (3)
Aorta		3.0 ± .4 (4)*	*6.6 ± .7 (6)*	*7.4 ± .1 (4)*
Skin		7.3 ± .8 (3)	*15.3 ± 1.8 (4)*	*20.8 ± 2.8* "
Uterus		.8 ± .1 (2)	*3.3 ± .9 (3)*	*3.2 ± .5* "

* See footnote, Table I.

TABLE III. Influence of Age on Content of Elastin (g of Elastin/100 g of Tissue) in Female Rat.

Tissue \ Age Group	3-5 wk A	8 mo B	2 yr C
Upper leg muscle	.09 ± .05 (4)	.04 ± .02 (6)	.03 ± .02 (3)
Lower " "	.07 ± .04 (2)	.06 ± .03 "	.04 ± .03 "
Abdominal "	.06 ± .04 (4)	.05 ± .02 "	.03 ± .01 "
Kidney	.26 ± .25 "	.04 ± .01 (5)	.04 ± .02 "
Lung	.33 ± .20 "	.32 ± .12 (7)	.30 ± .03 "
Liver	.03 ± .02 "	.04 ± .01 (6)	.01 ± .01 "
Spleen	.16 ± .11 "	.11 ± .07 (7)	.15 ± .13 "
Tendon	.13 ± .03 (6)	.26 ± .11 "	*.30 ± .09 (9)*
Heart	.16 ± .11 (3)	.10 ± .08 (4)	.06 ± .01 (3)
Aorta	6.14 ± .36 (4)*	5.93 ± .56 (6)	6.14 ± 1.15 (8)
Skin	1.31 ± .33 (5)	.60 ± .27 (10)	.41 ± .16 (7)
Uterus	.11 ± .03 (3)	.35 ± .35 (8)	.25 ± .16 (9)

* See footnote, Table I.

and sex, no change was observed in elastin content of upper leg, lower leg or abdominal musculature, or in kidney, liver, spleen and uterus. In tendinous tissue, elastin increased with age, with virtually the entire change taking place during first 8 mo of life. A consistent decrease in elastin occurred with age in connective tissue of hearts of both sexes. In female rats, there were no demonstrable changes with age in amount of elastin present in lungs. However, in the male, there was a decrease from 3-5 wk to 8 mo and no further decrement up to 2 yrs of age. More elastin was observed in male than in female aorta at 8 mo of age. More elastin was present in skin of females than in that of males at 3-5 wk of age (Table VI).

Non-scleroprotein (Table IV). Calculations for nonscleroprotein were made in 4 tissues only: tendon, aorta, skin and uterus. No significant changes with age or sex were observed in values of non-scleroprotein for aorta nor with age for uterus. In tendon and skin, non-scleroprotein decreased with age in both sexes and more dramatically in the former tissue.

Total collagen (Table IV). In both sexes, there was an increase in total collagen of tendon up to 8 mo of age. At all ages from fifth week onward, the amount of collagen present in male tendon was significantly greater than that in the female (Table VI). Total collagen content of *aorta* increased steadily with age in both female and male rats from third wk to eighth mo. No significant change occurred thereafter. There was an increase in total collagen with age in skin and uterus of all animals.

Percentage of soluble collagen in total collagen (Table V). In uterus and tail tendon,

TABLE IV. Influence of Age on Total Non-scleroprotein and Total Collagen (g of Protein/100 g of Tissue) in Female Rat.

Tissue \ Age Group	3 wk A	4 wk A-1	5 wk A-2	8 mo B	2 yr C
Total non-scleroprotein tissue					
Tendon	10.8 ± 1.4 (4)	8.6 ± .1 (2)	10.2 ± 1.5 (3)	*5.0 ± 2.1 (4)*	*3.0 ± 1.2 (4)*
Aorta	5.7 ± 1.1 " *	6.9 ± 1.1 (3)	5.5 (1)	6.5 ± .7 (4)	6.5 ± .8 (4)
Skin	7.2 ± .7 (3)	6.2 ± .4 (4)	10.6 ± .6 (3)	*8.1 ± 1.3 (4)*	*5.7 ± .8 (4)*
Uterus	7.3 ± .0 (2)	6.0 ± 2.5 "	9.6 ± 5.4 "	7.9 ± 1.2 (3)	10.4 ± 3.0 (4)
Total collagen					
Tendon	12.5 ± 1.4 (4)	14.3 ± 1.2 (2)	12.7 ± .6 "	*20.3 ± 1.1 (4)*	*19.2 ± 1.2 (4)*
Aorta	5.0 ± .4 " *	6.0 ± .9 (3)	5.5 (1)	*7.8 ± .8* "	*8.1 ± .9* "
Skin	7.3 ± 1.7 "	*10.1 ± 1.6 (4)*	*10.8 ± .9 (3)*	*20.6 ± 2.1* "	*24.6 ± 2.9* "
Uterus	2.3 ± .1 (2)	2.3 ± .9 (4)	2.2 ± 1.1 (3)	*4.5 ± 1.0 (3)*	*4.3 ± .6* "

* See footnote, Table I.

TABLE V. Influence of Age on Percentage of Soluble Collagen in Total Collagen (g of Soluble Collagen/100 g of Total Collagen) in Female Rat.

Age	3 wk	4 wk	5 wk	8 mo	2 yr
Group	A	A-1	A-2	B	C
Tissue					
Tendon	85.6	82.1	*68.0*	*48.8*	*18.6*
Aorta	37.4*	29.2	36.6	*17.6*	*9.0*
Skin	70.0	*45.1*	*32.0*	*25.7*	*14.0*
Uterus	65.8	*42.8*	26.8	*28.1*	*26.0*

* See footnote, Table I.

aorta and skin of both sexes, the percentage of soluble collagen in total collagen decreased with age. At 3 wk of age, more soluble collagen was present in tail tendon of female than of the male. At 8 mo a significantly higher percentage of soluble collagen in total collagen was found in the skin of females than of males (Table VI).

Comment. These data show that in both sexes tissues rich in scleroprotein (tendon and skin) undergo greater changes in protein composition than tissues low in scleroprotein. In general, in these tissues there is a decrease in non-scleroprotein with aging and a concomitant increase in scleroprotein. In other tissues, with a few exceptions, levels of protein fractions were relatively constant from 3 wk to 2 yr of age. With increasing age the percentage of soluble collagen in the scleroprotein fraction decreased and amount of insoluble collagen increased with age. The third component of the scleroprotein fraction, elastin, remained relatively constant in all tissues except tendon in which there was an increase with age. The principal differences between sexes were that in skin, tendon and aorta, the insoluble collagen was higher in young females while at 8 mo males had higher levels. After this time there were no differences between the sexes. The data indicate that the aging process in terms of changes in scleroprotein occurs principally in tendon, skin, uterus and aorta.

Summary. Tissues from tail tendon, aorta, skin, uterus, lung, muscle, heart, liver, kidney and spleen of male and female rats at 3-5 wk, 8 mo and 2 yr of age were fractionated into soluble protein, insoluble collagen and elastin. Amount of soluble collagen in the soluble protein fraction was calculated according to amount of hydroxyproline. Significant changes in these fractions with age occurred only in skin, tendon, uterus and aorta, with scleroprotein increased and non-scleroprotein decreased.

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TABLE VI. Sex Differences* in Composition of Connective Tissue.

		Tissue value (g/100 g)							
		Abdominal muscle		Tendon		Aorta		Skin	
Constituent	Age change observed	♂	♀	♂	♀	♂	♀	♂	♀
Soluble protein	3-5 wk			26.5	18.5				
	8 mo							8.3	13.4
Insoluble collagen	3-5 wk	.6	1.5	2.2	4.5			5.3	7.3
	8 mo	.5	1.7	22.9	10.3	8.2	6.6	22.0	15.3
Elastin	5 wk							.5	1.3
	8 mo					7.4	5.9		
Total collagen	5 wk			17.6	12.7				
	8 mo			27.0	20.3				
	2 yr			25.1	19.1				

* These are statistically significant differences within 5% level of confidence.

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Nature and Source of Appendical Secretion.* (24865)

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Demonstration that the vermiform appendix of man(1), chimpanzee(2), and rabbit (3) secretes fluid at pressures approaching systolic blood pressure has provided a basis for understanding the pathogenesis of obstructive appendicitis in man. However, because of the simplicity of treatment of appendicitis by surgery, there has been little stimulus for study of the site or mechanism of this fluid production. The following experiments were undertaken to determine the cellular elements responsible for this process in the vermiform appendix. Normally the rabbit appendix secretes from 10 to 56 ml of a slightly cloudy, colorless liquid of specific gravity 1.001-1.006 and pH 8.1-8.5 in 6 hour period.

Method. Adult albino rabbits of both sexes were utilized. Measurement was made of volume of secretion and secretory pressure with appendix ligated at base and attached to a collecting balloon or manometer respectively. Several experiments were designed to modify or nullify participation by certain cellular elements: I. *Suppression of lymphoid elements.* a) Exposure doses of x-ray in range of 250-1000 R were delivered to a series of appendixes.† Volume and pressure measurements were made 2 to 21 days thereafter. b) Cortisone was given parenterally in dosage of 5 mg/day for 7 and 14 days and 12.5 mg/day for 14 days. This steroid causes dissolution of lymphocytes and actual decrease in weight of lymphoid elements(4). II. *Stimulation and exhaustion of mucous cells.* a) Pilocarpine(5) in 2.5 mg dosage was administered hourly for 4 hours prior to fluid collection and in another group of animals the drug in same dosage was given hourly during a 6-hour collection period. b) Mustard oil(6) in olive oil was instilled into appendical lumen for 6 hours and fluid collection and pressure measurements carried out thereafter. III. *Destruction of surface epithelium.* a) Silver nitrate in 2 and 5% concentrations was instilled into the appendix for 5 minutes prior to pressure and volume measurement. IV. To determine if a cellular process requiring oxygen utilization was actually involved, sodium cyanide M/600 in 2 ml amounts was placed in appendical lumen and pressure determined(7). Appropriate control volume and pressure measurements were made. Histologic examination was made of all appendixes studied. Specimens were fixed in formalin and stained with hematoxylin and eosin or fixed in absolute alcohol and stained by Mayer's mucicarmine technic.

Results. Volumes of fluid secreted by the appendix under the experimental conditions are shown in Table I. Following X-irradiation, there was significant decrease in volume of fluid produced, both in early post-irradiation period and after 3 weeks. Sections of irradiated appendixes uniformly demonstrated severe suppression of lymphoid elements; those appendixes showing great reduction in secretory volume uniformly demonstrated epithelial damage as well. Irradiation was quite

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† For irradiation a G. E. Maximan x-ray machine was operated at 140 KV, 15 MA with no added filter (H.V.L. = 3.7 mmAl). Using 27 cm F.S.O. exposure dose rate was 232 roentgens/minute as measured with Victoreen r-meter.

TABLE I. Effect of Several Agents on Secretion by Rabbit Appendix.

No. of animals	Agent administered	Time from treatment to fluid collection	Collection period (hr)	Vol of section (ml) Mean	Range
6	None (control)	—	48	122	95-138
7	X-irradiation (1000 r)	21 days	48	36	10- 52
5	*Idem*	48 hr	48	45	28- 82
10	X-irradiation (250-750 r)	72 "	48	72	27-200
9	None (control)		6	23	10- 56
5	Cortisone, 5 mg/day, 7 days		6	5	3- 15
6	*Idem* 14		6	13	8- 25
7	Cortisone, 12.5 mg/day "		6	11	6- 20
5	Pilocarpine, 2.5 mg/30 min. for 8 doses (before collection)		6	21	11- 29
6	Pilocarpine, 2.5 mg/30 min. for 12 doses (during collection)		6	13	10- 23
5	Mustard oil (4 drops/25 ml olive oil) for 6 hr		6	24	13- 33
5	Mustard oil (15 drops/25 ml olive oil) for 6 hr		6	18	5- 26
4	Mustard oil (25 drops/25 ml olive oil) for 6 hr		6	26	15- 40
6	$AgNO_3$, 2% for 5 min.		6	15	10- 30
4	*Idem* 5%		6	11	3- 20

uniformly followed by increased number of mucous secreting goblet cells both of surface epithelium and crypt walls.

Administration of cortisone was followed by reduction in thickness of lymphoid layers to approximately one-half to one-third of normal with dissolution of considerable numbers of lymphocytes. Although there was decreased volume of fluid following cortisone administration, the decrease is significant only when the drug was given in 5 mg amounts daily for one week.

All animals given pilocarpine had extreme salivation and repeated defecation during period of administration. There is no significant difference in volume of fluid produced compared with control animals whether the drug was given before or during collection period. Mucicarmine stained sections of most appendixes showed no histologically demonstrable effect of pilocarpine on mucous cells. However, appendixes of 2 animals receiving the drug before, and of 3 rabbits receiving pilocarpine during fluid collection, showed definite increase in mucus production by goblet cells of crypt walls. This greater secretory activity by mucous cells was not associated with increased volume of fluid secretion in any of these animals.

The mucous membrane of the appendix was exposed to irritant effects of mustard oil in 3 concentrations. Erosion of surface epithelium and edema and hemorrhage of subepithelial tissues occurred in a few specimens so treated. Exhaustion of goblet cells of surface epithelium occurred in a few, but the majority of appendixes showed no erosion, edema or hemorrhage, and normal to increased amounts of mucus being produced by goblet cells of surface and crypt walls. In none, including those specimens showing hypersecretion by goblet cells, was there significant change in volume of secretion. Significant decrease in fluid production followed instillation of 5% $AgNO_3$ into the appendix, but not following use of this substance in 2% concentration. Organs so treated demonstrated fairly uniform damage to surface epithelium. In several specimens, coagulation necrosis of surface epithelium led to obliteration of crypt orifices and, presumably, as a result of continuing secretion, crypts were converted into cystic structures with complete pressure obliteration of the papillae.

Following application of NaSCN, no change from baseline intraluminal pressure occurred during 5 hours. In all other groups in which intraluminal pressure was determined, values

of 50 to 100 cm of water pressure were observed. These pressures are of the same magnitude as those obtained with control animals.

Discussion. Although total destruction of lymphoid elements of the appendix was not observed in these studies, suppression of these elements *per se* did not appear to modify appendical secretion significantly. Rather, decrease in secretion appeared to be related more to damage of epithelial elements than of lymphoid follicles.

The goblet cell apparently is not responsible for the large volume of secretion occurring in rabbit appendix. Stimulation of goblet cells was produced in several experiments with pilocarpine and mustard oil as evidenced in mucicarmine stained sections. In none of these animals was there an increased volume of secretion. Irradiation led to increase in numbers of goblet containing cells of surface epithelium and crypt walls, but to decreased rather than increased secretion.

Obliteration of papillae with conversion of crypts into globular cyst-like spaces following closure of crypt orifices suggests that secretion occurs at least in part in these crypts. Since cells of crypt walls appear to be predominately mucous cells, and since these do not appear to contribute significantly to the secretory process, epithelial cells overlying the papillae must be responsible. The role of surface epithelium and subepithelial elements was not clarified in these experiments. However, as it was possible to destroy surface epithelium by silver nitrate and have secretion continue, the epithelial elements of the papillae must contribute significantly to the process.

Summary. 1. Large volumes of fluid produced by vermiform appendix of the rabbit, result from a cellular process requiring oxygen utilization. 2. Suppression of lymphoid elements of the appendical wall produces no significant decrease in secretory volume or pressure. 3. Stimulation of mucous cells is not associated with augmented secretion. 4. Destruction of surface epithelium reduces but does not abolish secretion. It would appear that secretion stems primarily from epithelial elements overlying papillae in crypts.

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ACTH *in vitro* on Release of Nonesterified Fatty Acids from Adipose Tissues of Adrenalectomized Rats. (24866)

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The role of hormones in regulation of fat mobilization is not clear. During fasting plasma nonesterified fatty acids (NEFA) are released from fat depots and are transported to other tissues(1). It has been shown that factors affecting concentration of NEFA in plasma similarly affect their release from adipose tissue incubated *in vitro*(2,3). Addition of ACTH to incubating medium enhances release of NEFA from adipose tissue(4). Injection of this hormone into animals causes an increase in hepatic lipids which is prevented by adrenalectomy(5,6,7,8). Presumably, increased hepatic lipid is due to higher rates of mobilization of fat from adipose tissue(9). Thus, it is possible that the augmented level of liver lipids found in ACTH treated animals is due partially to increased release of NEFA from adipose tissue and transport of these fatty acids to the liver where they are esterified(10). The purpose of this investigation was to determine whether in rats, previous adrenalectomy had an effect on release of NEFA by epididymal adipose tissue, on incu-

TABLE I. Effect of Adrenalectomy on NEFA Release from Epididymal Rat Adipose Tissue.

Animal treatment	No. of animals	μM NEFA released/g adipose tissue/3 hr
None	4	2.86 ± .69*
Adrenalectomy + water	6	.55 ± .20
" + saline	6	.66 ± .06

* Mean ± stand. error of mean.

bation *in vitro*, and the response of this tissue to added ACTH.

Material and methods. Sprague-Dawley, male, albino rats, weighing 150-250 g were adrenalectomized in one stage under ether anesthesia, given 1% sodium chloride or tap water and fed Purina Fox Chow. They were weighed daily. Each group contained 4 to 6 animals. On fifth postoperative day, and after 24 hour fast, the animals were given sodium pentobarbital and decapitated. Epididymal fat bodies were removed, weighed on Roller Smith Torsion balance and incubated at 36°C for 3 hours with shaking in 4 ml of Krebs Ringer phosphate buffer containing 5% bovine albumin(2). Each epididymal fat body served as control for the contralateral tissue to which ACTH was added. ACTH (ACTHAR, 10 units/ml, lot # 10808, Armour Labs) was diluted with Krebs Ringer phosphate buffer and added at concentrations of 25 and 2.5 milliunits/ml of incubation medium. NEFA was measured at beginning and end of incubation period. Analysis was based on modification of extraction procedure described by Dole(11), using isooctane rather than heptane and washing the isooctane with dilute sulfuric acid as described by Gordon(1). Two ml of incubation medium were placed in conical glass stoppered centrifuge tubes containing 8 ml of a mixture of isopropyl alcohol and 3 N sulfuric acid (40:1); 4 ml of water and 10 ml of isooctane were added. Tubes were shaken for one minute, allowed to stand for approximately 15 minutes, then centrifuged at 300 x g for 5 minutes. The top layer was transferred to another glass stoppered centrifuge tube and 9 ml of approximately 0.002 N sulfuric acid was added; tubes were shaken for 30 seconds, centrifuged for 5 minutes at 300 x g and the bottom layer removed. The washing procedure was repeated once with 0.002 N sulfuric acid and twice with distilled water. After final wash, the tubes were centrifuged and 5 ml aliquots were removed from the isooctane fraction. Titration was carried out in 12 ml conical centrifuge tubes as described by Gordon(1). The results are expressed as μM of NEFA released/g adipose tissue (wet weight) during 3 hour incubation. Experiments in which palmitic acid was added to plasma in concentration of 2.65 meq/liter indicated 96-100% recovery. Lactic acid when added to plasma to a concentration of 1 N did not appear in the washed isooctane extract.

Results. Incubation of epididymal adipose tissue from normal rats causes a significant release of NEFA into the incubation medium (Table I). The amount of NEFA released/g of adipose tissue is markedly decreased by adrenalectomy; no significant difference was found between animals maintained on tap water or 1% sodium chloride (Table I). This finding was confirmed in 3 experiments.

Addition of ACTH at concentrations of 25 and 2.5 milliunits/ml of incubation medium caused a highly significant increase in NEFA released from adipose tissue of normal animals. Three times as much NEFA was released into the medium/g of tissue with 25 milliunits as with 2.5 milliunits of ACTH (Table II). Following adrenalectomy, addition of 25 milliunits ACTH elicited about one-half the NEFA released by adipose tissue of normal animals whereas 2.5 milliunits had no significant effect. There was no difference between adrenalectomized animals receiving tap water or saline (Table II) in the release of NEFA at either dose of ACTH.

TABLE II. Effect of ACTH on Release of NEFA from Adipose Tissue of Normal and Adrenalectomized Animals.

Animal treatment	Milliunits ACTH/ml	No. of animals	μM NEFA released/g tissue/3 hr*
None	25	6	13.14 ± .81†
Adrex + water	"	5	5.49 ± .93
" + saline	"	5	6.08 ±1.32
None	2.5	6	3.75 ± .76
Adrex + water	"	6	.41 ± .25
" + saline	"	6	.16 ± .01

* Differences between NEFA release of treated tissues and contralateral controls.

† Mean ± stand. error of mean.

Discussion. It has been suggested that circulating NEFA, in part, is the means by which fatty acids are transported from depots to active tissues in the fasting state(12). Dole's results on NEFA composition(13) and Gordon's studies on arteriovenous differences indicate that the source of circulating NEFA is adipose tissue(1). That starvation, carbohydrate feeding, epinephrine, and insulin influence release of NEFA from adipose tissue *in vitro*(2) in a manner compatible with the effects observed *in vivo*(1,11) support the theory that plasma NEFA, during fasting, originates from adipose tissue. The view that fatty acids released on incubation of adipose tissue result from hydrolysis of tissue esterified fatty acids is based on the finding that the amount released exceeds the initial concentration of NEFA in the tissue. The observation that mono- and diglycerides, hydrolysis products of triglycerides, are increased in adipose tissues following injection of epinephrine (which increases NEFA mobilization) supports the idea that plasma NEFA originates in adipose tissue and are derived from the triglycerides(14).

Our results indicate that hydrolysis of esterified fatty acids present in adipose tissue, occurs to a smaller extent in adrenalectomized animals than in normal animals. During fasting less fat is found in livers of adrenalectomized animals than normal, indicating less mobilization(5). This is what would be expected from the *in vitro* results.

Adrenalectomy in our experiments diminished markedly or abolished the increased release of NEFA from adipose tissue resulting from varying amounts of ACTH. These results are compatible with the finding that adrenalectomy prevented the increase in hepatic lipids in animals given ACTH(5,6,7,8).

White and Engel(4) reported that *in vitro,* minimum concentration of ACTH necessary to demonstrate a lipolytic effect in adipose tissue from normal animals was greater than that required to stimulate adrenal gland *in vitro* or *in vivo.* They suggested that these results would be expected if ACTH specifically stimulated the adrenal gland. On the other hand, our results show a significant lipolytic effect demonstrable with 1/50th the concentration reported by White and Engel. This concentration of ACTH is well within the range reported for *in vitro* effects of ACTH on adrenal tissue(15). Since ACTH causes release of NEFA from adipose tissue in absence of adrenals, in which the hormone is active *in vitro,* it appears that ACTH has an independent lipolytic effect on adipose tissue.

Thus, it is possible that ACTH has a physiological role in regulation of fatty acid mobilization.

Summary. 1. Adrenalectomy decreases release of nonesterified fatty acids (NEFA) from rat epididymal adipose tissue. 2. Addition of small amounts of ACTH *in vitro* to adipose tissue increases release of NEFA. This effect is markedly diminished in adrenalectomized animals. 3. It is suggested that ACTH may have a physiological role in fatty acid mobilization not mediated by adrenal cortical tissue.

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Effects of Parietalectomy and Sustained Temperature on Thyroid of Lizard, *Sceloporus occidentalis.** (24867)

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Stebbins and Eakin(1) observed that lizards (*S. occidentalis* and *Uma inornata*) maintained in laboratory runways exhibited heightened thyroid epithelium and reduction of colloid several months after removal of the parietal eye (parietalectomy). A lesser but similar effect has been found in a sample of their field specimens of *S. occidentalis* and is here reported.

Methods. A total of 377 animals was treated surgically, half parietalectomized, half sham-operated, and returned to study area in Berkeley hills(1). Because the principal objective of the investigation was an analysis of effects of parietalectomy on behavior, recaptured animals were released immediately after inspection and recording of certain data; only toward the close of the study were specimens taken for microscopical examination. Accordingly, the sample of 22 parietalectomized and 12 sham-operated lizards was small. Fig. 1 shows relative heights of thyroid epithelium in summer (June-July), spring (March), and winter (November) animals, parietalectomized (*P*) and sham-operated (*Sp*), expressed as percentage of follicular diameter. The mean value for each group (indicated by cross lines in the Dice-Leraas graphs) was based upon measurements with ocular micrometer of average epithelial height and follicular diameter of 20 randomly selected follicles in each specimen. The mean heights in μ of the follicular epithelium of the classes are: summer—*P* 10.6, *Sp* 10.4; spring *P* 9.4, *Sp* 6.3; winter—*P* 4.8, *Sp* 4.5.

Results. Fig. 1 shows no difference between parietalectomized and sham-operated animals taken in the summer. Although differences between experimental and control animals collected in spring and winter were not found by a t-test to be statistically significant at the 5% level, they are in the same direction as that observed in our laboratory experiments(1), namely, toward a higher follicular epithelium in the parietalectomized lizards.

Considering the annual picture of both parietalectomized and sham-operated animals, it is clear that there is a significant seasonal variation in microanatomy of the thyroid gland. Lizards taken in November possessed numerous large follicles filled with dense colloid. The epithelium was commonly no higher than the diameter of its flattened nuclei. The thyroid gland of specimens collected in March exhibited a large number of small or medium-sized follicles, the epithelium of which was low columnar. The follicular epithelium in summer specimens was high, sometimes 3 times the diameter of its nuclei, and the colloid was reduced and thin. This seasonal variation in thyroid gland of *S. occidentalis,* recently shown also by Wilhoft(2), is basically similar to that described by Miller (3) in *Xantusia vigilis.*

Could above changes be caused by annual cycle in solar heat? Eggert(4) found that hypertrophy of the thyroid occurred in *Lacerta agilis* kept at temperature of 24-29°C. Wilhoft(2) has shown similarly that maintaining *S. occidentalis* at 35°C (mean of normal activity range(5) for a few weeks leads to hypertrophy of the thyroid. The following experiment was performed to determine if this effect is mediated by the pituitary. Adult *S. occidentalis* freshly collected from Berkeley hills in summer were hypophysectomized under cold anesthesia (animal placed in cracked ice). By means of dental drill a small hole was made in the floor of the cranium immediately ventral to the hypophysis. The anterior lobe was removed by aspiration; in some instances posterior lobe also was extirpated. A sham-operation in controls consisted of drilling through the cranium and

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exposing the hypophysis. Animals were operated in sets of 2 or 3, matched so far as possible as to size and sex. In sets of 2, a hypophysectomized (*H*) and a sham-operated (*Sh-H*) animal were placed in separate containers in the 35°C chamber for 3 weeks. In sets of 3, in addition to *H* and *Sh-H* lizards, a second sham-operated animal (*Sh-R*) was maintained for 3 weeks in a room with variable temperature, never below 14° at night and above 35°C in the day (see Wilhoft(2) for details of method). The numbers of specimens were: 20 *H*, 20 *Sh-H*, and 10 *Sh-R*.

The mean percents (epithelial height/follicular diameter) are shown in the 3 Dice-Leraas graphs on the right of Fig. 1. The group of sham-operated animals kept at room temperature (*Sh-R*) was unfortunately reduced in number by death of 4 animals, the result of over-heating when thermal control failed. It will be observed, however, that there is good agreement between thyroids of these controls and those of summer specimens in the field study (Fig. 1). Mean epithelial heights in μ of the classes are: *Sh-R* 12.7; *Sh-H* 20; *H* 3.8. Histological differences between the 3 groups were strikingly significant. Without first checking the identity of a specimen, one could almost invariably assign it to the proper class by inspecting a few representative thyroid follicles. The hypophysectomized lizards usually ceased eating (mealworms) after a week or 2 and became inactive. Five died before the end of 3 weeks. Sham-operated animals at 35° (*Sh-H*), on the other hand, fed well and were very active.

Discussion. The studies on parietalectomy of *S. occidentalis* reported by Stebbins and Eakin(1) and in this paper suggest that removal of the third eye leads to varying degrees of thyroid stimulation depending upon environment of animal. The effect is more apparent in animals maintained in laboratory runways, with lower thermal limit in the daytime often at 25°C, than in field specimens which had considerably lower temperatures available to them even on the warmest summer day. Wilhoft(2) has shown that keeping this lizard at mean temperature of its normal activity range for a few weeks induces hypertrophy of the thyroid, and in this paper we

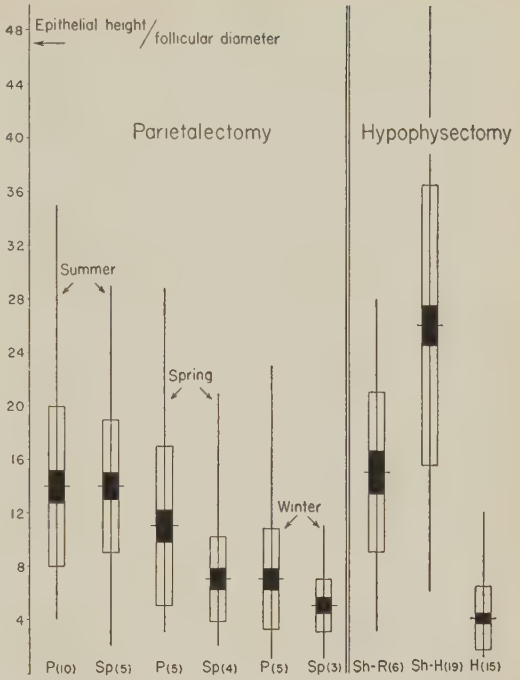

FIG. 1. Height of thyroid epithelium in *Sceloporus occidentalis*, expressed as percentage of follicular diameter, in field study on parietalectomy (left) and laboratory study on hypophysectomy (right). In each Dice-Leraas graph cross line indicates mean percent and vertical line the range of percent in each sample (P = parietalectomized, Sp = sham parietalectomized, H = hypophysectomized, Sh-R = sham hypophysectomized at room temperature, Sh-H = sham hypophysectomized at higher temperature (35°C). Solid rectangles show 2 stand. errors of mean and open rectangles stand. dev.

have presented evidence that this response is mediated by the anterior lobe of the pituitary body, presumably by its thyrotropic hormone. Recently, Fortune(6) found that high temperature (25°C) increased secretion of thyrotropin in the teleost, *Phoxinus laevis*. Taking these several findings together it seems probable that the parietal eye, hypophysis, and thyroid are somehow linked together in the physiology and behavior of this lizard in relation to solar radiation. The difference in degree of effect of parietalectomy upon the thyroid of field and laboratory animals is probably owing to the fact that in laboratory runways thermal conditions—especially a lower day-time thermal limit as high as 25°C—to which the animals were subjected over several months, intensified the differential (yet to be specifically identified) between experimental

and control animals. In the field, on the other hand, because climatic factors are more complex and variable and thermal range available is so much greater, the differential is not sufficiently magnified to bring about a clear dissimilarity in histology of the thyroid of parietalectomized and sham-operated lizards. A possible explanation for the similarity in thyroids of experimental and control lizards collected in summer might be a masking of the differential by the endocrine interplay incident to reproduction.

Summary. 1. Parietalectomized lizards (*Sceloporus occidentalis*), free living in the field, presented evidence of above-normal thyroid activity in animals collected in winter and spring, but not in summer. 2. An annual cycle of thyroid activity is exhibited by both parietalectomized and sham-operated lizards. 3. Thyroid hypertrophy induced by 3 weeks of constant temperature (35°C) is mediated by the hypophysis.

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Cytomegalic Inclusion Disease of Lacrimal Glands in Male Laboratory Rats.* (24868)

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This paper reports the presence of cytomegalic inclusion disease in intraorbital and exorbital lacrimal glands of laboratory rats. This condition was observed in adult males of 8 laboratory colonies but was not seen in any females nor in wild urban or rural Norway rats.

Methods. For recent toxicological study (unpublished) several hundred adult male Sprague-Dawley rats were purchased from a Midwestern firm. Routine histological survey of tissues from head and neck showed invasion by cytomegalic inclusion disease. Certain distinctive features described by Wyatt (1) as essential to complete a morphological diagnosis were satisfied, namely, eosinophilic intranuclear inclusion bodies, margination of nuclear chromatin, and specific cellular gigantism. Basophilic cytoplasmic inclusions were not observed. To procure normal lacrimal tissue, 6 adult male rats from each strain of Naval Medical Research Inst. stock, *i.e.*, Long-Evans, Sprague-Dawley, and NMRI-Dental caries-susceptible, were selected at random and lacrimal glands prepared for histological examination. All strains showed extensive lacrimal involvement by inclusion disease. All other organ systems were free of any cytomegalic inclusion disease. Sample male rats, 4 to each group, were procured from 4 other laboratory colonies in various geographic locations in U.S. Inclusion disease was present in all lacrimal glands, although variations in inclusion morphology and severity of the lesion were observed. These findings will be reported later. A group of wild urban male and 6 rural Norway rats, trapped in the Baltimore area, were free of lacrimal involvement. However, urban rats possessed intranuclear inclusions within kidney tubular cells, similar to the condition previously reported by Hindle(2). Salivary gland virus disease as described by Kuttner (3) and Smith(4) was present in acinar epithelium of submandibular glands and in ductular cells of sublingual glands of farm rats.

Results. Sample male and female rats of

* Opinions or assertions contained herein are those of the writers and are not to be construed as official or reflecting the views of Navy Dept. or naval service at large.

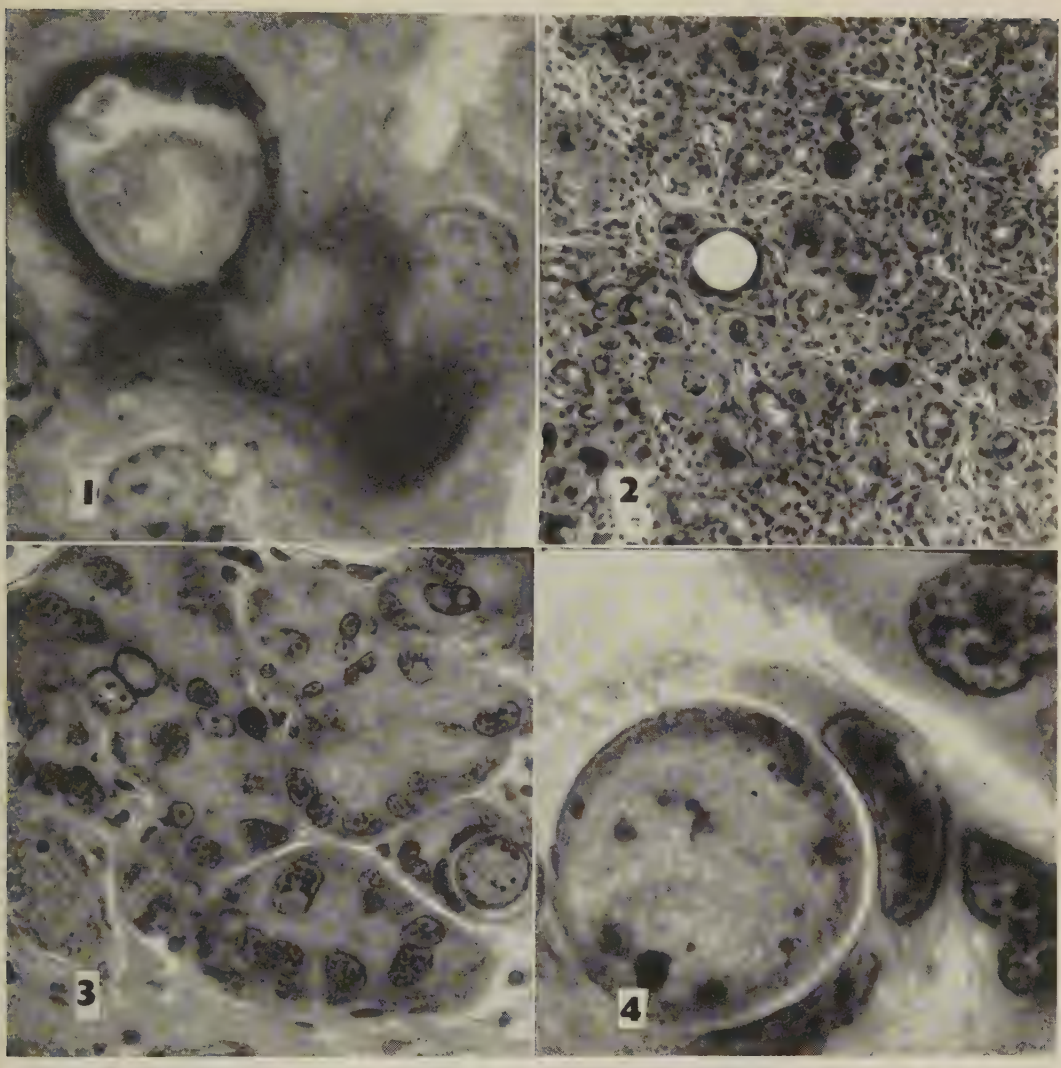

FIG. 1. Cytomegalic inclusion disease in exorbital lacrimal gland. Note diagnostic intranuclear acidophilic inclusion body with typical halo and margination of nuclear chromatin. NMRI "D" strain, 350-day male. 800 ×.

FIG. 2. Cytomegalic inclusion disease involving intraorbital lacrimal gland of a 350-day-old male, NMRI-D caries susceptible strain showing bizarre changes in acinar morphology. × 190.

FIG. 3. Atypical inclusion morphology in exorbital lacrimal gland of 250-day-old Sprague-Dawley male rat. × 250.

FIG. 4. Enlarged eosinophilic inclusion body 23 μ in diameter enclosed by cytomegalic ductular cells. Note compression of adjacent nuclei. Oil immersion, 800 ×.

NMRI-D strain were examined at 50-day intervals starting at birth, to record progressive changes in lacrimal pathology during disease. A typical diagnostic eosinophilic intranuclear inclusion body is shown in Fig. 1. In Fig. 2, progressive characteristic cellular changes are recorded from intraorbital lacrimal gland of a 350-day-old male. Affected acinar cells exhibit marked pleomorphism and subsequent cytomegaly. Bizarre nuclear patterns, namely, asymmetrical multipolar mitotic figures, syncytial masses, and giant hyperchromatic club-shaped nuclei containing one to 4 eosinophilic inclusions, simulate neoplastic morphology. The interstitial tissue shows an apparent increase in stromal cells and lymphocytic infiltration. Both intraorbital and exorbital lacrimal glands exhibited the same apparent de-

gree of involvement. In 450- and 500-day-old males, much of the degenerated lacrimal acinar tissue had been replaced by sebaceous type acini similar in morphology to the Harderian gland. The presence of intranuclear inclusions and subsequent bizarre cellular activity was not observed in any females regardless of age.

Many atypical inclusion bodies (Fig. 3) were observed in 200-day Sprague-Dawley males. The spherical eosinophilic mass shown in Fig. 4 appears to be a giant pleomorphic inclusion body measuring 23 μ diameter.

Although the significance of the disease and method of treatment are not known, it would seem essential that frequency of occurrence in other laboratory strains from different geographic locations be determined.

Summary. Cytomegalic inclusion disease of intraorbital and exorbital lacrimal glands was observed in adult male rats from 8 laboratory colonies but was not seen in any females nor in wild urban or rural Norway rats. Affected acinar cells showed characteristic eosinophilic intranuclear inclusion bodies and subsequent cytomegaly. Bizarre nuclear patterns simulated neoplastic morphology in late stages of the disease.

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Lack of Correlation Between Adrenal Weight and Injury in Grouped Male Albino Mice.* (24869)

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Grouping previously segregated mice results in adrenal hypertrophy which is least in dominant animals and greatest in most subordinate animals(1). Adrenal hypertrophy also occurs in freely growing populations of house mice, although fighting may be rare in these populations(2). Apparently neither fighting nor injury accounts for the adrenal hypertrophy(3). Although this belief has not been tested critically, it resulted from 2 types of observation: (a) adrenal hypertrophy apparently occurs in populations without fighting as well as in those with fighting, and (b) dominant animals usually fight more than subordinate animals. However, evidence has not been presented eliminating the possibility that injury from fighting could account for adrenocortical hypertrophy. This paper presents data showing lack of relationship between injury from fighting and adrenal weight in grouped male mice.

* Opinions expressed in this article are those of the author and not necessarily those of the Navy.

Methods. These data on adrenal weight and scarring were collected from experiments exploring relationship between adrenal weight and population density. All data are from grouped mice whose adrenals averaged 8 to 10% heavier than those of their segregated controls. These comparisons between adrenal weights of grouped and segregated mice are discussed in detail elsewhere(3,4,5,6,unpubl.). The data for each group of means or for each Table represent a collection of homogeneous data with respect to treatment, experiment, and time. Albino male mice from Naval Medical Research Inst. colony were used. Procedure was similar for each experiment and no groups were subjected to experimental procedures other than grouping. Male mice were segregated at weaning and for 3 weeks thereafter then were grouped by different sizes for 1 week, then autopsied, and severity of scarring recorded for each mouse. In some experiments scarring was recorded as absent, few scars, or severely scarred, while in other

experiments the actual number of scars was either counted or designated as 20 or more scars.

Results. When 40 populations were divided into those with no scarred mice and those with 1 or more mice scarred from fighting, the amount and severity of scarring presumably from fighting did not affect adrenal weight (Table I). Absence of scarring is indicative of either very little or no fighting.

Mice from 26 populations of 4, 5, or 6 "fighting" males each were categorized scarring absent, slight, or severe (more than 6 scars) and mean adrenal weight determined for each category of scarring for each population. Thus mean adrenal weight and standard error for each scarring category was calculated. Mean adrenal weights were respectively 5.86 ± .16, 5.56 ± .18, 5.58 ± .18 for unscarred, slightly scarred, and severely scarred categories. Twenty-three of the 26 populations had one or more unscarred mice. When there was only 1 unscarred mouse in a population (17 of the 23 populations) it was the dominant mouse in that population. There was an apparent tendency for adrenal weight to decrease with increased scarring, although the differences in mean adrenal weight were not significant ($P>0.30$).

Table II summarizes data from experiment with 10 populations of 6 mice each. Number of scars on each mouse was counted after one week of grouping. There was a significant decline in absolute adrenal weight and body weight as scars/mouse increased. However, adrenal weight relative to body weight did not decline, and in fact remained constant or even increased as scars increased, although the differences in mean relative adrenal weights between categories of scarring were not significant.

TABLE I. Difference in Mean Adrenal Weights of Albino Mice from Groups in Which There Was Fighting with Injury (Cuts about the Rump, Tail, Hind Legs) and from Groups from Same Experiments in Which There Was No Fighting or Fighting without Injury. Group mean values used. The 2 experiments were run at different times and are not comparable.

Exp.	Fighting in groups	Group size	No. of groups	Mean adrenal wt ± stand. error
I	No injury	6	16	5.29 ± .12
	Injury	6	14	5.29 ± .10
II	No injury	4	3	6.00 ± .31
	Injury	4	7	5.80 ± .23

TABLE II. Adrenal Weights *vs* Number of Scars for 60 Mice from 10 Groups of 6 Each. A barely significant difference ($P<.05$) exists in absolute adrenal weights between those with no scars and those with more than 20. There is also a significant decline in weight with increased scarring. Adrenal weights relative to body weights do not vary significantly with respect to scarring. Relative adrenal weight tends to increase with increased scarring.

		Means ± stand. error		
Scars	Mice	Absolute adrenal wt	Body wt	Adrenal wt, mg/100 g body wt
0	17	5.33 ± .17*	25.2 ± .62	21.3 ± .65
1– 4	16	5.40 ± .18	24.4 ± .85	22.6 ± 1.10
5– 8	9	5.21 ± .23	23.8 ± .70	22.0 ± 1.22
11–17	7	4.90 ± .26*	22.4 ± .85	22.1 ± 1.65
>20	11	4.78 ± .20*	21.1 ± .58	22.9 ± 1.35

* $P<.05$.

Discussion. Although there is usually a relationship between degree of scarring and social rank in individual mice of a group, the relationship between adrenal weight and social rank(1), as indicated by amount of scarring, is not clearly apparent. However, there is a suggestion that adrenal weight relative to body weight increases with increasing scarring within groups. This tendency for relative adrenal weight to reflect increased scarring must come from differences in social rank rather than degree of injury, as there was no difference in mean adrenal weights of groups with and without scarred mice (Table I). These results warrant the conclusion that scarring (or injury) from fighting is not an important factor in producing adrenal hypertrophy observed in grouped mice. These results are a further indication that sociopsychological interactions between mice, and not physical factors, are responsible for adrenal hypertrophy following grouping.

The reason for lack of correlation between adrenal hypertrophy and injury seems to be that injuries from fighting are quite superficial. The wounds usually penetrate only the skin, heal rapidly, and therefore do not represent serious injury. Nevertheless, an occasional mouse is attacked so severely by its cage mates that wounding may be extensive. Healing is usually slow in these cases and involves considerable scarring. However, gross

damage of deeper tissues has not been observed in these mice. It might be expected that these mice would show a more marked adrenal hypertrophy, but this cannot be evaluated with present data.

Summary. Amount of scarring and adrenal weight were compared for 280 male albino mice from 50 populations of 4, 5, or 6 mice each. No relationship was found between adrenal weight and absence or presence of scarring or between adrenal weight and amount of scarring. There was a significant decline in absolute adrenal weight with increased scarring, but this decline disappeared when adrenal weights were corrected for body weight. It was concluded that adrenal hypertrophy after one week of grouping did not reflect amount of injury received.

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Effects of Adrenalectomy and Aldosterone on Sodium Concentration in Renal Medulla of Hydropenic Rats.* (24870)

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Relying upon the hairpin countercurrent theory proposed by Hargitay and Kuhn(1), Wirz suggested that concentrating ability of mammalian kidney depends on water-free active solute reabsorption taking place in the ascending limb of the loop of Henle(2). This theory has been supported by the demonstration in hydropenic animals of increasing osmotic pressure in the medulla, from its cortical margin to the extremity of the papilla (3,4) and by recent work of Gottschalk and Mylle showing high osmolality of tubular fluid obtained from the tip of the loop of Henle(5). Removal of solutes from the fluid flowing up the ascending limb of the Henle loop is suggested by the hypotonicity of urine obtained from the first portion of the distal tubule(2,6,7). Quantitatively, the major ion involved in active transport process upon which the concentrating mechanism depends, is likely to be sodium(6). In man, aldosterone causes, within 2 to 4 hours, a reabsorption of sodium by kidney greater than could be accounted for by exchange for potassium and hydrogen ions (Crabbé, J., unpublished observations), thought to occur in the distal convoluted tubule(8). This suggested that aldosterone might stimulate sodium transport system in the loop of Henle. Assuming that sodium is the solute actively reabsorbed at that site and relying upon the observation that its proportional contribution to total solute content of the papilla is constant(4), it should therefore be possible to examine this hypothesis by measuring concentration of sodium in the papilla of concentrating kidney.

Methods. Twenty-four Holtzman female rats, 4 months old, were deprived of water 40 hours, and fasted 28 hours, before sacrifice. Two groups of 6 rats (groups B and D) had been adrenalectomized under ether anesthesia 80 hours before start of experiment, *i.e.,* 120 hours before sacrifice, and had been maintained on food and 1% sodium chloride solution *ad lib.* during the interval. Twelve intact rats were placed on same regimen (Groups A and C). The animals were transferred to metabolic cages 28 hours before sacrifice.

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† Fellow of Jane Coffin Childs Memorial Fund for Medical Research.

‡ Markle Scholar in Medical Science.

TABLE I. Sodium, Potassium and Water Content of Renal Tissue/100 g Dry Weight in Rats.

		Papilla			Cortex		
		Na	K	H_2O, ml	Na	K	H_2O, ml
		meq			meq		
Intact (Group A)	Mean	98.7	34.7	520	25.7	32.1	247
	S.D.	10.2	3.3	64	2.5	1.4	28
Adrenalectomized (Group B)	Mean	102.3	41.0	693	26.1	36.3	306
	S.D.	22.4	7.5	150	4.4	3.7	35
Intact + aldosterone (Group C)	Mean	95.8	32.5	481	23.8	32.0	243
	S.D.	11.6	3.8	39	1.3	1.1	12
Adrenalectomized + aldosterone (Group D)	Mean	104.1	41.0	705	27.8	36.9	307
	S.D.	29.7	15.2	181	6.3	5.6	62

TABLE II. Concentrations of Sodium and Potassium in Tissue Water of Rats.

		Papilla		Cortex	
		Na	K	Na	K
		meq/l			
Intact (Group A)	Mean	193.7	66.7	106.0	130.1
	S.D.	38.5		10.7	
Adrenalectomized (Group B)	Mean	149.6	59.1	84.3	118.5
	S.D.	27.8		17.3	
Intact + aldosterone (Group C)	Mean	200.2	67.4	98.1	131.9
	S.D.	29.4		6.0	
Adrenalectomized + aldosterone (Group D)	Mean	147.3	58.1	90.6	120.5
	S.D.	11.0		9.5	

Each rat of Groups C and D was given subcutaneous injections of 20 μg of d-l aldosterone monoacetate§ in 0.02 ml of ethanol diluted to 0.1 ml with isotonic saline, 28 and 16 hours before death, and twice that dose 4 hours before sacrifice. Those of Groups A and B received only ethanol diluted in saline. All animals were exsanguinated from abdominal aorta under intraperitoneal Nembutal anesthesia. Kidneys were quickly excised and dissected with scissors; the inner part of medulla, consisting largely of papilla, could readily be identified and isolated due to its white appearance. Both papillae of each rat were weighed in stoppered tared tubes, dried 24 hours at 105°C, reweighed, ground, extracted with 0.75 N nitric acid for 48 hours at room temperature, and the tubes centrifuged before removal of supernatant for sodium and potassium measurement by internal standard flame photometry. Pairs of slices of renal cortex were handled similarly.

Results. Results of tissue analyses, expressed as meq of sodium and potassium and as ml of water/100 g of dry tissue, are summarized in Table I. Neither adrenalectomy nor aldosterone had any significant effect on sodium content/100 g of dry solids of either renal papilla or cortex. Adrenalectomy resulted in increase in potassium content of both papilla and cortex, of 18% and 13% respectively. This was not affected by administration of aldosterone. The most striking change following adrenalectomy was in degree of hydration of renal tissue. The water content of the papilla increased 33%, while the cortex increased 24%. Once again aldosterone was without influence on these values.

In contrast to uniformity of sodium concentration/unit dry tissue weight, the concentration of sodium in tissue water (Table II) showed considerable change in both parts of the kidney, as a result of changes in tissue water content. Adrenalectomy caused a fall of 23% in papillary sodium concentration ($p = 0.05$) and of 20% in sodium concentration of the cortex ($p < 0.02$). Aldosterone again was without effect. Potassium concentration in the papilla and cortex tissue water was

§ Kindly supplied by Dr. R. Gaunt, Ciba Pharmaceutical Products, Summit, N. J.

likewise lowered by adrenalectomy whether aldosterone was given or not.

Discussion. The operation of the countercurrent multiplier concentrating mechanism depends upon simultaneous presence of 3 factors: 1) antidiuretic hormone, to render renal tubule permeable to water, 2) active, water-free reabsorption of sodium in the ascending limb of Henle's loop, and 3) adequate flow of blood in capillary loops of medulla. An attempt was made to influence the sodium transport mechanism, while maintaining amount of antidiuretic hormone maximal by prolonged dehydration and assuming that blood flow in medullary capillaries remained comparable in all animals. Under these conditions any change in concentration of sodium in renal papilla of animals could be ascribed to a change in rate of active sodium transport. The papillae of adrenalectomized rats contained lower concentrations of sodium in tissue water than those from normal animals. This confirms the results of Guinnebault and Morel(9) and supports the view that adrenal secretions are required for adequate function of countercurrent system.

Change in sodium concentration in papilla following adrenalectomy was entirely the result of changes in water content. Dry tissue solids represent a far better base of reference for such analyses, since it can be safely assumed that they remain constant. In consequence, one can determine whether changes in sodium concentration in wet tissue are due to changes in hydration or in sodium accumulation.

In light of countercurrent multiplying theory, in presence of antidiuretic hormone even a small change in rate of sodium transport is bound to result in much larger variations in tissue hydration. This dominance of water change over sodium content has also been observed when antidiuretic secretion alone is changed(10).

Changes in water content of renal cortex which followed adrenalectomy were similar but less pronounced than those seen in the papillae. The significance of this is not clear.

Reasons why administration of aldosterone in relatively large amounts to adrenalectomized animals, failed to return the composition of renal papilla to normal, may be manifold. Duration of aldosterone treatment was probably sufficient since this hormone produces sodium retention within 2 to 4 hours, and changes observed in electrolyte content of serum and muscle of these rats (Borle, A. B.), *et al.*, unpublished observations). Perhaps aldosterone exerts its sodium-retaining effect elsewhere in the renal tubule. A second possibility is that other adrenal steroids may be required for aldosterone to increase sodium concentration in the medulla. This latter hypothesis is supported by clinical observation that glucocorticoids are required to enable a patient with Addison's disease to handle a water load in normal fashion(11,12).

The failure of aldosterone to produce noteworthy changes in renal tissue composition of normal rats is not so surprising. The conditions of these experiments were such that a maximal retention of sodium due to endogenous secretion of hormone might be expected (13,14) and thus addition of further exogenous hormone would have little effect.

Summary. 1. Water, sodium, and potassium contents of renal cortex and papilla have been measured in normal and adrenalectomized hydropenic rats with and without administration of exogenous aldosterone. 2. Sodium content of renal papilla and cortex expressed in terms of dry tissue solid were little changed by adrenalectomy of aldosterone. However, water content was increased by adrenalectomy, the increase being 33% in papilla and 24% in the cortex. Aldosterone had no significant effect on this phenomenon. 3. It is suggested that the adrenal secretion exerts an influence on active transport of sodium across the ascending limb of loop of Henle. 4. Under our conditions, aldosterone is not sufficient to restore renal tissue concentration of sodium to normal in adrenalectomized animals.

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Hyperlipemia and Hemolysis. II. Interaction of Sodium Oleate with Human Erythrocytes in Homologous Plasma. (24871)

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Previous studies(1) on the interaction of sodium oleate with washed human erythrocytes suspended in buffered isotonic saline indicate that erythrocyte-oleate binding precedes hemolysis; once lytic quantities are bound, repeated iced saline washings fail to dissociate oleate or check hemolysis, and following lysis, hemolytic substances are released. The present studies consider the interaction of sodium oleate with washed human erythrocytes suspended in homologous plasma. This investigation is a continuation of studies concerned with intermediate pathways for *in vivo* hemolysis reported during conditions associated with rapid increases in serum unesterified fatty acid(2-5).

Methods and materials. Sodium oleate (Baker) was employed as the fatty acid; all solutions were prepared in sodium phosphate buffered saline, ionic strength 0.15. The pH of buffered oleate solutions, measured with glass electrode, was 7.10 at 37°C and 7.20 at 25°C. All oleate solutions remained at room temperature 24 hours prior to use since hemolytic potency declined upon standing, rapidly at first, then more slowly. Mechanical fragility of human erythrocytes suspended in fresh heparinized plasma or in buffered saline was determined at 25°C with pipette shaker generating 2 inch horizontal strokes at 220/minute; all suspensions were tested in 1 x 12 cm test tubes. On completion of shaking, the tubes were spun at 2500 rpm, 0°C, 15 minutes and supernatant hemolysis determined by a Klett-Summerson photoelectric colorimeter (550 mu. filter).

Results. Binding of oleate by plasma. Ability of mammalian plasma and plasma proteins to bind fatty acid and neutralize hemolytic activity has been documented extensively (6-9). The present data are consistent with these findings. The assumption is made that human plasma inhibition of oleate hemolysis is dependent on plasma oleate binding. Table I indicates capacity of bovine serum albumin (Armour, Fraction V) to render exogenous oleate hemolytically inert; 1 mole of bovine serum albumin binds the hemolytic equivalent of 5 to 6 moles exogenous sodium oleate at pH 7.1, 37°C. These results do not reflect *total* oleate binding capacity of bovine serum albumin since initial oleate albumin levels were not determined. Lysis inhibition by bo-

TABLE I. Capacity of Bovine Serum Albumin to Bind Exogenous Sodium Oleate.

	Exogenous sodium oleate ($\times 10^{-7}$ moles)	Bovine serum albumin* ($\times 10^{-7}$ moles)
	24	4.6
	12	2.3
	6	1.1
	3	.5
Mean	11	2.1

* Minimum quantity required to prevent oleate lysis of washed human erythrocytes at 37°C, pH 7.1. Each value is mean of 3 determinations.

TABLE II. Inhibition of Oleate Hemolysis by Human Plasma.

2.5 ml sodium oleate pretreated with .10 ml of:	Final oleate conc. ($\times 10^{-4}$ M) 4		1	
	37°C	25°C	37°C	25°C
Isotonic buffered saline	1.1*	1.5	2.0	5.0
Homologous human plasma	60	60	60	60
Idem, albumin	4.0	24	60	60
" globulin	1.8	3.2	10	30

* Time (min.) for 100% hemolysis after addition of 0.5 ml aliquots of buffered saline containing 5×10^5 washed human erythrocytes to 2.6 ml of oleate preparations. Each value is mean of 3 determinations.

vine serum albumin was not increased by prior incubation with oleate, confirming reported rapid fatty acid binding by serum albumin (10). Oleate hemolysis inhibition was also consistently obtained with normal human plasma and the corresponding plasma albumin and globulin fractions prepared by ammonium sulfate fractionation as described previously (11); typical findings are given in Table II. It is stressed that to inhibit hemolysis, plasma or plasma proteins must be added to oleate *prior* to insertion of washed erythrocytes; when added after erythrocyte-oleate contact, plasma accelerates hemolysis(11).

Firmness of human plasma-oleate binding was tested by studying the effect of mechanical agitation on washed human erythrocytes

TABLE III. Effect of Oleate on Mechanical Fragility of Human Erythrocytes in Fasting Homologous Plasma in 4 Subjects.

	Fasting homologous plasma		Fasting homologous plasma + sodium oleate (8×10^{-6} moles oleate/1 ml plasma)*	
	Shaking time (min.)			
	0	180	0	180
	.02†	.02	.02	.32
	.04	.05	.05	.45
	.03	.04	.04	.81
	.02	.04	.04	.96
Mean	.03	.04	.04	.63

* This concentration of sodium oleate in buffered isotonic saline produces 100% hemolysis at 25°C in 2 min. without mechanical agitation.

† % hemolysis of 0.5 ml washed packed erythrocytes suspended in 2 ml plasma. Each value is mean of 3 determinations at 25°C. Hemolysis determinations on non-shaken erythrocyte suspensions were performed after 180 min.

suspended in fasting homologous plasma and in fasting homologous plasma pretreated with 8×10^{-6} moles sodium oleate/ml. The results (Table III) suggest that human plasma oleate binding *in vitro* is not dissociated appreciably by mechanical agitation. Thus 3 hours of vigorous shaking of human erythrocytes suspended in fasting homologous plasma increased mean lysis from 0.03 (control) to 0.04%; despite pretreatment of plasma with sufficient oleate to lyse 100% of unshaken erythrocytes in isotonic saline within 2 minutes (at comparable pH and temperature), hemolysis increased from 0.04 (control) to but 0.63%. Sufficient exogenous oleate was therefore dissociated by shaking to account for only 0.58% hemolysis. The diminutive

TABLE IV. Effect of Oleate on Mechanical Fragility of Human Erythrocytes in Buffered Isotonic Saline in 4 Subjects.

	Control erythrocytes		Oleate pretreated erythrocytes*	
	Shaking time (min.)			
	0	60	0	60
	.03†	.19	.19	.74
	.03	.20	.15	1.13
	.04	.10	.10	.46
	.02	.15	.14	.63
Mean	.03	.16	.15	.74

* 0.5 ml packed washed erythrocytes suspended in 2.5 ml sodium oleate, 0.5×10^{-4} M, pH 7.2, 25°C and then immediately spun at 2500 rpm, 0°C for 3 min. followed by 5 buffered saline washings at 0°C, pH 7.2. Control erythrocytes treated identically except buffered saline substituted for oleate.

† % hemolysis of 0.5 ml packed erythrocytes suspended in 2 ml isotonic buffered saline (pH 7.2). Each value is mean of 3 determinations at 25°C. Hemolysis determinations on non-shaken erythrocyte suspensions were performed after 60 min.

quantity of oleate dissociated was further appreciated upon finding that oleate increases not only static but also mechanical fragility of human erythrocytes (Table IV). It is probable therefore that even less exogenous oleate was dissociated by mechanical agitation than would be required for 0.58% hemolysis in a static system.

Competitive oleate binding by human erythrocytes and homologous plasma. The preceding experiments concern hemolysis inhibition when oleate is *pretreated* with human plasma. Hemolytic activity of oleate

TABLE V. Competitive Oleate Binding by Human Erythrocytes and Homologous Plasma.

Lytic system	Hemolytic system (Plasma + sodium oleate) + erythrocytes	Hemolytic system (Plasma + erythrocytes) + sodium oleate
1	4.5*	.15
2	7.5	.40
3	7.5	.60

* Minimum sodium oleate ($\times 10^{-6}$ moles) required for detectable hemolysis at 37°C, pH 7.4. Each value is mean of 3 determinations.

upon *simultaneous* contact with plasma and erythrocytes was determined. Sodium oleate was added in increasing quantities to a series of tubes containing 0.6 ml normal fasting human heparinized plasma at 37°C. To a parallel series of tubes containing 0.6 ml aliquots of the same plasma, 0.4 ml packed washed homologous erythrocytes were added and all tubes mixed by inversion. Within subsequent 3 minutes, sodium oleate was added to tubes containing erythrocytes and erythrocytes were added to tubes containing oleate. All tubes were inverted 5 times immediately after each addition. Composition of the 2 series of tubes at completion of mixing was identical, the sole difference being that varying quantities of oleate were permitted to react with human plasma at 37°C in presence and absence of homologous erythrocytes. All tubes were left undisturbed at 37°C for 2 hours. The minimal quantities of sodium oleate producing visible hemolysis were then recorded. Typical results are given in Table V. The results suggest significant competition between plasma and homologous erythrocytes for binding *exogenous* oleate. By this mechanism, *in vitro* oleate hemolysis protection by human plasma can be reduced 30-fold.

Discussion. Addition of bovine serum albumin or of human plasma, plasma albumin or globulin to oleate solutions prior to insertion of washed human erythrocytes inhibits subsequent hemolysis. This fatty acid hemolysis inhibitory effect has long been recognized(6-9). If the assumption is correct that plasma inhibition of oleate lysis is dependent on oleate binding, then once oleate is bound *in vitro* by human plasma, the binding is firm. This conclusion is based upon the observation that 3 hours of vigorous mechanical agitation of human plasma containing sufficient exogenous oleate to lyse 100% of unshaken erythrocytes within 2 minutes in isotonic saline at pH 7.4 resulted in but 0.63% hemolysis; 0.58% hemolysis was attributable to exogenous oleate. Precise quantitation of oleate dissociated during mechanical agitation was not possible from hemolysis measurements since oleate increased erythrocyte mechanical fragility; the actual quantity of oleate dissociated during mechanical agitation would therefore not be comparable to that required for similar hemolysis in static systems. It could be argued that large quantities of oleate *were* dissociated from human plasma and interacted with erythrocytes during mechanical agitation but plasma inhibited lytic consequences ordinarily expected in saline media. Recent observations indicating that human plasma does not inhibit but intensifies hemolysis following oleate-erythrocyte interaction(11) precludes this possibility.

In preceding experiments, oleate was added to human plasma followed by insertion of washed erythrocytes. Obviously, erythrocytes were exposed solely to oleate the plasma was incapable of binding. Such systems generally have been the type studied previously, from which marked fatty acid hemolysis inhibitory action of mammalian plasma has been inferred. However, when erythrocytes are suspended in homologous plasma *prior* to oleate addition, the hemolysis protective potential of plasma wanes. If this finding is correlated with the preceding data, it appears that whenever exogenous oleate enters whole human blood *in vitro,* the red cells and plasma compete for binding oleate; that fraction of exogenous oleate bound by plasma is rendered hemolytically inert whereas that fraction bound by the erythrocyte constitutes the hemolytically active moiety. The oleate hemolysis protective potential of normal human fasting plasma can be reduced 30-fold by such a mechanism.

Summary and conclusions. Normal human plasma binds exogenous sodium oleate firmly *in vitro* as indicated by inhibition of oleate hemolysis despite vigorous mechanical agitation. The oleate hemolysis inhibition can be reduced 30-fold by adding oleate directly to

whole blood, rather than to plasma subsequently reconstituted as whole blood. Whenever oleate enters whole human blood *in vitro,* red cells and plasma compete for binding oleate; that fraction of oleate bound by plasma is rendered hemolytically inert whereas that fraction bound by erythrocyte constitutes the hemolytically active moiety.

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Pharmacologic Studies of a New Antihypertensive Compound N-(o-Methoxyphenyl)-N′-(3-methoxypropyl)-piperazine Phosphate (HT1479) (24872)

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In screening of compounds for antihypertensive activity certain members of a series of piperazines showed significant power to produce a fall of blood pressure, both in normotensive and hypertensive animals. Further testing of this group (all synthesized by Sommers *et al.* of Organic Chemistry Dept., Abbott Labs) yielded one (HT1479) which possessed satisfactory antihypertensive characteristics, and promised to excell in one important phase, that of relatively prolonged duration of action in anesthetized animals.

Methods. All injections were given intravenously unless otherwise specified. Blood pressure was recorded from carotid artery of cats, dogs and monkeys anesthetized with phenobarbital-pentobarbital mixture, and recorded with ink-writing mercury manometer. Blood pressure was also recorded from femoral artery of trained unanesthetized normotensive dogs. Cats were used for studies on carotid reflexes, effect of anoxia and nictitating membrane activity. Carotid sinus occlusion was achieved by 2 pneumatic cuffs which applied pressure to the carotids *in situ,* with minimum of trauma or traction. Anoxia was produced by connecting tracheal cannula to spirometer containing 100% nitrogen. Nictitating membrane experiments employed direct mechanical recording of membrane movements. The preganglionic portion of the superior cervical nerve was stimulated by square wave or 60 cycle impulses. Standardized doses of epinephrine were given before and at intervals after administration of piperazine compound. Duration and degree of inhibition of pressor response was used as semi-quantitative measure of relative potencies. Parasympatholytic action was determined in anesthetized cats by comparing depressor response to standardized doses of acetylcholine (1 μg/kg) before and after administration of drug. Antihistaminic potency of compounds was evaluated in anesthetized cats by their influence on depressor response to standardized dose of histamine (0.25 μg). Renal hypertensive rats, prepared by Grollman method (1) were used to study hypotensive effect of the compounds. Blood pressure was measured in these unanesthetized rats by photoelectric method of Kersten *et al.*(2). Direct effect on heart was studied in the Langendorff heart preparation, perfused at pressure of 40-50 cm with Ringer-Locke solution. The drugs

were added to the perfusing solution. Heart contractions were recorded with ink-writing level, and coronary outflow was registered continuously by modified Stephenson volume recorded(3). Acute toxicity was determined in mice. LD_{50} and standard errors were calculated by log-probit method of Miller and Tainter(4). Chronic toxicity studies were carried out in rats and dogs.

Results. Anesthetized dogs and cats. HT1479 in dose of 0.5 mg/kg produced a lowering of blood pressure of 55-70 mm Hg. At the end of 6 hours, blood pressure was still 30 mm below usual normal level after this period. At 250 μg/kg there was a fall of 40-50 mm with return to normal in about ½ hour in dogs and cats. 250 μg/kg administered intraduodenally produced a decline beginning in 7 minutes and progressing gradually to 30 mm below normal. This level was sustained over 30 minutes before slowly returning to normal. In the above dose range, the heart rate slowed, but seldom as much as 10%. In 28 animals many other tests in the range from 25-250 μg/kg produced results comparable to those already mentioned. In the pithed, vagotomized cat, doses of 75 and 100 μg/kg intravenously caused prolonged drops of blood pressure and a slight inhibition of epinephrine response. One mg/kg produced complete reversal of 5 and 10 μg of epinephrine. In both species bilateral occlusion of renal blood flow prolonged depressor effect up to 100%. When occlusion was terminated the pressure rose above control level. In anesthetized bilaterally nephrectomized monkeys depressor effects were similar to those osberved in cats or dogs with occlusion of renal artery except that duration of effect was much prolonged but magnitude of initial response was about the same.

Langendorff heart preparation. The effects of HT1479 and one of its homologues, HT507, on the isolated heart were studied in 22 cat hearts, 10 monkey hearts and 2 rabbit hearts. When mixed with Ringer-Locke solution in concentrations from 0.25 to 10 μg/ml and perfused through coronary circulation, they produced no consistent effect except a slight decrease or increase in coronary flow. There

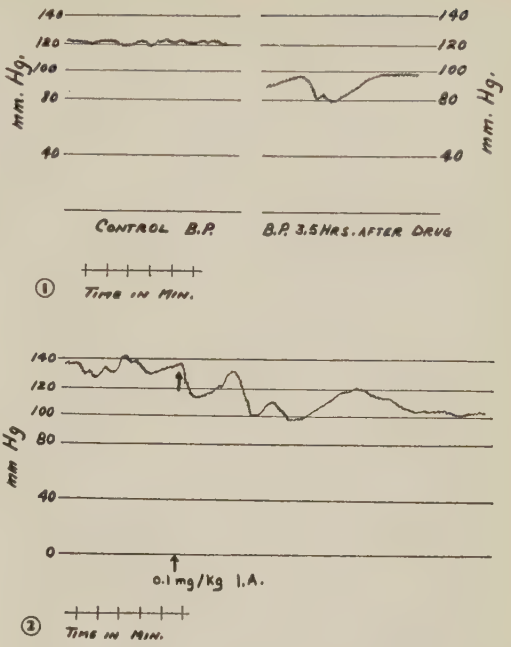

FIG. 1. Effect of HT1479 on blood pressure of an unanesthetized normotensive dog given 5 mg/kg orally.

FIG. 2. Effect of HT1479 on blood pressure (femoral artery) of an unanesthetized normotensive dog after 0.1 mg/kg given intra-arterially into femoral artery.

were no significant alterations in heart rate or amplitude of contractions.

Coronary Flow in the Dog. In 5 experiments using a Morawitz cannula(5) the coronary flow of anesthetized dogs was directly measured. Intravenous (femoral) infusion of 0.1 to 1 mg/kg of HT1479 caused a slight increase in coronary flow. When 10 mg (approximately 1 mg/kg) was injected directly into the vena cava there was a slight decrease in flow. In either case the change was less than 15%.

Unanesthetized normotensive dogs. A single dose of 5 mg/kg orally produced lowering of blood pressure of 16 mm Hg when determined after 3 hours in one dog and of 20 mm after 3.5 hours in another dog (Fig. 1). A dose of 0.1 mg/kg into femoral artery produced immediate lowering of 25 mm in the artery of this extremity (Fig. 2). Electrocardiograms recorded following intravenous dose of 1 mg/kg showed no significant abnormalities. A compensatory increase in heart rate occurred from a control of 90 beats/minute to 120 at 2

TABLE I. Effects on the Anesthetized Animal.

Compound No.	Dose/kg I.V. (mg)	Effect on blood pressure mm Hg	Duration	Epinephrine effect	Nictitating membrane response	Serotonin effect
			Anesthetized cat			
HT1479	.1	−55	Returning to −20 sustained	60% inhibited	50% inhibited	Normal
	1	−70	80 min.	Reversed	80% "	40% inhibited
507	.25	−40	60 "	Normal	Normal	
			Spinal cat			
HT1479	.075	−35	60 min.	Slightly inhibited		
507	.25	−55	Sustained			
			Anesthetized dog			
HT1479	.1	−20	Sustained	Absent		
507	.5	−45	60 min.	Normal		
			Anesthetized monkey			
HT1479	.05	−50	Returning to −20 sustained	Normal		
507	.5	−45	Sustained	"		Normal

minutes and 105 at 5 minutes. This change is probably attributable to fall in systemic blood pressure when the drug reaches general circulation.

Peripheral blood flow in the dog. Measurement of peripheral arterial blood pressure with Nolf triple-manometer preparation likewise indicated that intra-arterial injection of 25 μg/kg of HT1479 had a direct vasodilator effect. The dilator action was usually less than subsequent systemic response when the drug entered the general circulation. This confirms the observation in the unanesthetized dog.

Unanesthetized hypertensive rats. In a group of 6 chronic hypertensive rats with average blood pressure of 160 mm Hg (range 152-168) HT1479 in single dose of 0.5 mg orally (equivalent to approximately 2.5 mg/kg) produced a marked hypotensive effect. All animals showed significant decrease in blood pressure of about 25-30 mm, returning gradually to pretreatment levels in about 3 hours.

Miscellaneous effects. Blood pressure responses in anesthetized cats to intravenous injections of l-arterenol, acetylcholine and histamine were not modified by doses of this compound which reverses the response to epinephrine. Neither were typical responses of blood pressure to electrical stimulation to the central end of sciatic or caudal end of vagus nerves changed. The pressor effect of serotonin was reduced by about 40%. Doses of 0.5-1 mg/kg produced a 75% inhibition of the nictitating membrane response to superior cervical nerve stimulation. Carotid sinus reflex was 50-60% decreased and the response to anoxia reversed. In the expected therapeutic range (1-5 mg/kg) the compounds showed no potentiation of barbiturate sleep in mice. The same was true to 50 mg/kg orally. Table I shows activity of HT1479 and HT507 upon blood pressure and certain other functions. Tachyphylaxis does not occur in anesthetized cats or dogs.

Acute toxicity. The LD_{50} (Table II) of HT1479 was determined in mice, at least 10/level and analyzed by graphic method of Miller and Tainter(4). The symptomatology was entirely that of a depressant, with ataxia, depression and coma. Respiratory depression, slowed heart rate and terminal cyanosis were sequentially seen. At no time were convulsions observed. The depressant effects were seen only when the dose approached the lethal range. In dogs, HT1479 at doses up to 100 mg/kg subcutaneously produced no deaths, but doses of 120 mg/kg and above were lethal. A normal monkey was given 10 mg/kg of HT1479 by stomach tube. Only minimal behavioral effects were noted. There was a brief period of possible ataxia. The animal appeared tranquilized, he was drowsy, but

Nature and Appearance of Protein Digestion Products in Upper Mesenteric Blood. (24873)

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The long standing question of whether a protein meal is hydrolyzed completely to free amino acids before absorption into the portal blood seems to have been answered recently in the affirmative by Parshin and Rubel(1), Whipple and his co-workers(2), and by Denton and Elvehjem(3,4). Whipple, *et al.* reached their conclusion from the fact that C^{14} lysine fed to dogs was metabolized in precisely the same way regardless of whether it was free or bound in protein. The others based their conclusions on failure to find significant rises in peptide concentrations of portal blood plasma after protein meals. Many years ago, some workers(5) purported to find large amounts of peptides in portal blood of animals after protein meals, but this work suffered from the difficulty that there was no method available for specific analysis of individual amino acids or peptides. The approach was usually to measure amino nitrogen of a protein free filtrate before and after hydrolysis, or to measure the difference in amino nitrogen of plasma filtrates after precipitation with different reagents, *e.g.*, trichloroacetic acid and tungstic acid. Conclusions based on such methods are still being put forward(6). We made a direct search for peptides by newer methods, *i.e.*, ultrafiltration and ion exchange fractionation of all low molecular plasma amino compounds. There is no recent work using these methods which, to our knowledge, unequivocally demonstrates that chemically measurable peptides appear in portal blood plasma as a result of protein meal. We are not dealing here with minute amounts of peptides and whole proteins which must be absorbed to result in the well known immunological phenomena that occur in all animals. It has seemed reasonable, therefore, to conclude that proteins are digested with complete degradation to amino acids. Dent and Schilling(7) however, found no rises of free amino acids in portal blood of partially gastrectomized dogs fed dog plasma protein, from which they concluded that this protein not only escaped complete hydrolysis, but was absorbed whole, escaping even the peptide stage. They used paper chromatography and the hydrolysis procedure mentioned previously. Besides the fact that their dogs were partially gastrectomized, their experiments were marked with difficulties in getting their animals to accept the meal without immediate vomiting and diarrhea; when this occurred, these excreta were refed or eaten voluntarily.

Materials and methods. We attempted to recreate this apparently unique protein absorption process with normal, ungastrectomized dogs. They were prepared by cannulation of a branch of superior mesenteric vein by surgical technic of Denton, *et al*(8). We chose this vein, rather than the portal vein, to avoid admixture of gastric, splenic and large bowel venous blood. We believe that our study is unique in this respect. The catheters were kept open by daily irrigations with saline and heparin. In addition, we obtained lymph from one dog by cannulation of the thoracic duct. The dogs were allowed to recover for about a week after operation, and fed orally, or by stomach tube, about 70 g of cooked ground beef liver, or 30 g dry lyophilized homologous plasma protein. Plasma amino acids were fractionated and analyzed by the method of Moore and Stein(9,10). This method is uniquely suited for detection of peptides, since the smaller peptides are chromatographable on Dowex 50, and can be recognized among the familiar landmarks of known amino acids. In addition, they can be isolated and analyzed by hydrolysis and rechromatographing. We(11) isolated an acidic amino acid conjugate from plasma of nor-

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TABLE I. Micromols Amino Acid per 100 ml Superior Mesenteric Plasma Ultrafiltrate.

	Dog A (liver meal)			Dog B (liver meal)		Dog C (plasma meal)		
	Hr after feeding							
	0	3	6	0	6½	0	3	6
Taurine	4	10	8	5	30	3	5	5
Aspartic acid	0	1	1	4	14	1	3	14
Threonine	15	47	47	13.6	60	14	34	86
SGA*	42	50	44	78	91	48	87	130
Glutamic acid	8	10	9	10	9	7	6	31
Proline	13	20	60	13	69	16	26	48
Glycine	26	33	36	22	69	22	29	52
Valine	17	24	38	21	83	20	34	80
Methionine	3	13	11	6	28	4	3	11
Isoleucine	5	27	19	7	60	6	6	18
Leucine	9	47	37	15	91	9	47	101
Tyrosine	2	6	7	4	14	3	12	30
Phenylalanine	2	12	8	9	32	2	13	40
	Composition of conjugate							
Serine	0	0	0	0	0	0	2	0
Glutamic acid	7	0	0	0	6	2	8	0
Glycine	17	49	59	24	23	25	31	24
Alanine	0	3	6	0	0	0	0	0

* SGA = Serine + glutamine + asparagine.

mal human subjects in this way.

Results. Table I shows levels of mesenteric plasma amino acids in 2 dogs which received liver. Increases in amino acid concentrations are by no means uniform, and cannot be correlated with the composition of the fed protein.

We analyzed the lymph of one dog after a liver meal (Table II). The rises are similar to those of the mesenteric plasma.

The chromatograms showed no ninhydrin reactive components which could be peptides. We did, however, isolate an amino acid conjugate (supra) from each sample. These components gave no color with ninhydrin, and descended the columns rapidly, emerging ahead of aspartic acid. Subsequent hydrolysis in sealed tubes at 110°C for 12 hr, followed by rechromatography, yielded results shown in Table I. Glycine is the major component; we do not know the origin of these conjugates.

The effect of feeding homologous plasma protein is also shown in Table I. The rises of amino acids are, if anything, more dramatic than in liver fed dogs. No peptide fractions were found, nor was the conjugate remarkably elevated.

Discussion. Our results indicate that the normal dog digests homologous plasma proteins in the same way as other proteins, *i.e.*, with complete degradation to amino acids; the rises of free amino acids in the mesenteric plasma are at least as great in the dog fed plasma as in those fed liver. The increase in amino acid concentrations after meals followed no predictable pattern, nor did this process bear any relation to composition of the meal. The explanation for this may be that the amino acids have different absorption rates, that they compete with each other in the absorption process, or that their absorption is inhibited by nonprotein components of the diet. This last possibility was discussed by Denton and Elvehjem(3).

The amino acid conjugate, consisting mainly of glycine and glutamic acid was found in all plasma samples, and in lymph. We do not know the origin of this substance, nor the reason for its varying composition.

Fisher(12), theorizes that peptides are the basic units of protein synthesis, rather than free amino acids. If this is true, these peptides could not have a direct dietary origin, at least if liver or plasma are protein sources, nor does it seem from our experiments that amino acid conjugate(s) could arise directly from the diet.

Since our work was done, Newey and Smyth (13) have shown with *in vivo* experiments

TABLE II. Micromols Amino Acid/100 ml Thoracic Duct Lymph Ultrafiltrate.

	Dog C (liver meal)		
	Hr after feeding		
	0	2.5	4.5
Taurine	3	5.8	8.5
Aspartic acid	2	8	7
Threonine	13	27	27
SGA*	45	74	124
Glutamic acid	10	46	15
Proline	17	23	27
Glycine	23	32	35
Valine	24	28	23
Methionine	3	11	13
Isoleucine	6	14	15
Leucine	11	25	19
Tyrosine	5	11	12
Phenylalanine	5	20	18
	Composition of conjugate		
Serine	0		12
Glutamic acid	3		15
Glycine	7		8.5
Alanine	0		0

* SGA = Serine + glutamine + asparagine.

that a number of dipeptides introduced into the intestine of the dog appear in mesenteric plasma almost completely as free amino acids. Their results, then, are in general agreement with our own. Similar experiments done *in vitro* by Newey and Smyth confirm earlier work of Agar, *et al.*(14) which showed that dipeptides introduced on the mucosal side appeared on the serosal side almost entirely as free amino acids. The small and variable though definite passage of some peptide leaves open the possibility that such absorption might occur *in vivo* in cases of surgically altered or diseased gastrointestinal tract.

Summary. Dogs prepared with catheters in the superior mesenteric vein were fed meals of liver and homologous plasma protein. Analysis of the superior mesenteric blood plasma showed marked rises in amino acids up to 6 hours later. Digestion of the homologous plasma protein took essentially the same course as that of liver protein, contrary to results reported by others(7). No peptides were found and both proteins were apparently completely degraded to amino acids. Rises in lymph amino acids were qualitatively similar to those of the mesenteric plasma, though not as marked. An amino acid conjugate was isolated from all plasma samples, and its amino acid composition was determined. Its changing structure, and failure to respond in an obvious way to the meals leads us to believe that it is not of direct dietary origin.

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ECHO-9 Virus Antibody Titrations in a HeLa Cell System. (24874)

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The need to perform ECHO-9 virus antibody titrations during an outbreak of this infection in summer of 1957(1,2) prompted us to attempt the use of HeLa cells for these titrations because HeLa cells were more readily available to us than monkey kidney cells. HeLa cell adapted(3) ECHO-9 virus (Hill strain) was used as the antigen.* The results reported here clearly demonstrate that ECHO-9 antibody titrations can be readily executed in HeLa cell tissue culture.

Materials and methods. HeLa cells were grown in milk dilution bottles according to methods originally outlined by Syverton and Scherer(4). Lactalbumin hydrolysate-yeast extract medium (LHYE) containing 20% human serum was used as growth medium. After 7 days incubation at 37°C, bottles were scraped, cells dispersed in growth medium, a cell count made, and suspension prepared to dispense 50,000 to 60,000 cells/tube in 0.75 ml of growth medium. Tubes were incubated at 37°C for 3 or 4 days. On day tubes were used, the growth medium was removed, the cell sheet rinsed 3 times with Hanks' balanced salt solution (BSS) following which

* Seed virus was kindly supplied by Dr. Herbert A. Wenner, Univ. of Kansas School of Med., Kansas City.

0.9 ml of maintenance medium, consisting of a solution of LHYE containing 2% calf serum, was dispensed into each tube. *Virus pools.* One ml of a 10^{-1} dilution of HeLa-adapted ECHO-9 (Hill strain) virus was inoculated into each of several bottles of HeLa cells, in which cell sheets had been rinsed with Hanks' BSS and to which 10 ml of maintenance medium had been added. Bottles were incubated at 37°C, and when cell sheets showed maximum cytopathogenic effect (2 to 3 days after inoculation), they were placed at –20°C for storage. Virus pools were prepared by thawing and pooling stored tissue culture fluids. The fluids were then centrifuged at 1000 rpm for 10 minutes in horizontal centrifuge. The supernatant fluid subsequently used in neutralization tests was ampouled, and stored at –20°C. The virus suspension was titrated in half-log dilutions to determine $TCID_{50}$ of the virus for HeLa cells. Average $TCID_{50}$ for several lots tested was 0.1 ml of a $10^{-3.5}$ dilution. The dose was 100 $TCID_{50}$. *Serum specimens.* Acute and convalescent phase sera submitted in 1957 from persons with symptoms of acute central nervous system (CNS) disease (aseptic meningitis syndrome) were tested. A group of 48 pairs of serum specimens submitted in 1956, when Coxsackie B5 virus(5) infection was prevalent, was also tested. *Technic of test.* Serum specimens were diluted 1:4 with Hanks' BSS and heated at 56°C in waterbath for 30 minutes. Five additional 4-fold dilutions (through 1:4096) of serum specimens were prepared in Hanks' BSS. An equal volume of ECHO-9 virus suspension, adjusted to contain 100 $TCID_{50}$/0.1 ml, was mixed with each serum dilution and allowed to stand for one hour at room temperature. An inoculum of 0.2 ml of each serum-virus mixture was used/HeLa cell culture tube. A positive serum control was included in each series of tests. Virus titration, using 100, 10, and 1 $TCID_{50}$ of virus, in presence of negative human serum was also included to determine precise virus dose present in test. Serum neutralization endpoints were determined when half, or more than half of virus titration tubes inoculated with 1 $TCID_{50}$ of virus showed cytopathogenic effect. Titers were expressed as

Column Total →	294	77	54	33	7	1	1	Total 467
4096	1		2					3
1024	1			2	3		1	7
256	23	7	5	3	3	1		42
64	31	11	13	22	1			78
16	29	18	33	6				86
4	35	39	1					75
< 4	174	2						176
	< 4	4	16	64	256	1024	4096	

CONVALESCENT BLOOD TITER

ACUTE BLOOD TITER

FIG. 1. ECHO-9 antibody results on 467 pairs of acute and convalescent serum specimens. Titers are expressed as reciprocal of highest dilution of serum completely neutralizing 100 $TCID_{50}$ of ECHO-9 virus.

reciprocal of highest serum dilution which completely neutralized 100 $TCID_{50}$ of ECHO-9 virus. *ECHO-9 virus isolation and identification.* Monkey kidney cell tubes were purchased for ECHO-9 virus isolation and typing. Type-specific ECHO-9 antiserum was supplied through courtesy of Nat'l. Fn. for Infantile Paralysis.

Results. ECHO-9 virus neutralizing antibody titrations on paired serum specimens from 467 patients submitted in 1957 during outbreak of aseptic meningitis,† are presented in a correlation square, Fig. 1. The number in each cell represents number of persons who had the initial titer indicated below the square, and the convalescent titer indicated at left of square. Those persons represented in cells on diagonal line showed the same antibody titer in both specimens. The number of persons showing significant increase in titer is represented in cells appearing 2 or more squares above diagonal line. Specimens for virus isolation were available from 281 of 467 patients tested for antibodies and Table I shows relationship between isolation of ECHO-9 virus and antibody results.

† Subsequent studies revealed there were also 26 cases of Coxsackie B5 virus infection, and 64 cases of poliovirus infection to make the total aseptic meningitis experience in Minnesota during 1957.

TABLE I. Association of ECHO-9 Virus Isolation with Antibody Titer.

	No significant rise—Highest titer* observed				Significant (16× or >) rise in titer	Total
	<4	4	16	64 or higher		
Total patients	174	76	52	53	112	467
Patients from whom specimens were submitted for virus isolation	84	37	33	48	79	281
Patients from whom ECHO-9 virus was isolated	2	4	7	27	61	101
ECHO-9 isolation (%)	2.4	10.8	21.2	56.3	77.2	35.5

* Titers expressed as reciprocal of highest serum dilution completely neutralizing 100 $TCID_{50}$ ECHO-9 virus.

A significant rise in antibody was observed more often if first blood specimen was collected during first 6 days of illness. Among 112 patients showing significant rise, the date of onset was known for 106, and 87 of these were collected within first 6 days of illness. In contrast to this, 105 patients showed ECHO-9 antibody but no rise in titer. Date of onset was known for 100 of these, and 51 were collected within first 6 days of illness. Paired serum specimens available from 48 patients ill with CNS disease in 1956 were tested to compare incidence of ECHO-9 neutralizing antibody with the 1957 group. The highest antibody titer in this group was 64 in both specimens on one patient. One patient had a titer of 16 in both specimens, and 5 patients had a titer of 4 in both specimens.

Discussion. Fig. 1 shows that 174 (37%) of patients had no antibody in either serum specimen at dilution 1:4.

The data presented in Table I show that ECHO-9 virus was isolated from only 2, or 2.4% of patients in whom antibody was not detected at serum dilution of 1:4. Virus isolation rates increased with higher titers. The highest isolation rate (77.2%) was in the group in whom a significant (16-fold) rise in antibody titer could be demonstrated.

Monkey kidney cells were used for all ECHO-9 virus isolation and identification. All attempts to isolate ECHO-9 virus in HeLa cells were unsuccessful.

Titrations on paired sera from 48 patients with CNS disease in 1956 indicated that ECHO-9 antibodies were rarely demonstrable in that year since 41 (85%) of paired sera tested had no demonstrable antibody at serum dilution of 1:4.

Significance of the presence of antibody in instances in which a significant rise could not be demonstrated is not clear. In Fig. 1, convalescent titers are clustered at 4, 16, and 64. Fewer titers of 256 were demonstrated, while only a few persons showed titers of 1024 and 4096. In 29 patients showing a significant rise in antibody titer, the peak of antibody titer attained was 16. We interpreted this to mean that titers as low as 4 and 16 may be taken as supportive evidence for diagnosis of ECHO-9 infection under epidemic conditions. The higher virus isolation rates obtained in association with these antibody levels (Table I) support this view.

Summary and conclusions. 1) Use of a HeLa cell system, using HeLa cell-adapted ECHO-9 virus, has definite value in studying neutralizing antibody titers during an ECHO-9 epidemic. 2) 228 cases of CNS disease reported to our Department during summer and fall of 1957 were listed as ECHO-9 virus infections on the basis of clinical, epidemiological and/or laboratory findings. Laboratory evidence for support of the diagnosis was available in 214 cases.

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Experimental Attempt to Produce L-E Syndrome (Arthritis) in Swine with Hydralazine.* (24875)

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Lansbury and Rogers(1) reported syndromes resembling lupus erythematosis in patients receiving hydralazine (Apresoline) for hypertension. Attempts to produce this phenomenon in laboratory animals have failed (2,3). Pigs often develop a rheumatoid-like arthritis, probably of infectious etiology(4). If a triggering mechanism exists for a rheumatoid state as suggested in humans, we thought it might be activated in the pig by Apresoline.

Materials and methods. Ten litter mates of Poland-China breed pigs were used. Two served as controls and remainder treated with Apresoline. Intramuscular injections of Apresoline, exceeding dosages employed in humans, were used, given daily and increased weekly (Table I). Injections were stopped at end of 9 weeks because of physical difficulties. In addition, powdered Apresoline was added to their daily supplemental feedings in Karo syrup and a commercial pellet (containing Aureomycin, vitamins and minerals). When too much Apresoline was added, they refused it, thus it was possible to maintain an increasing dosage within palatable limits. Table I summarizes amounts of drug given orally and intramuscularly in correlation with increase of weight and age of the animals. Two experimental animals were sacrificed at 60 days and remainder at the end of fourth month. L-E preps., sheep-cell and Latex-fixation agglutination tests, as well as histological examination of synovial membranes, kidney, hearts and adrenals were performed.

TABLE I. Age, Weight of and Amount of Apresoline Administered.

Time (wk)	Avg wt (lb)	Apresoline I.M. (mg)	Apresoline Oral (mg)	Apresoline Daily total (mg)
1	10– 15	40	100	140
2	15– 20	80	200	280
3	20– 25	100	400	500
4	25– 30	"	600	700
5	30– 35	200	800	1000
6	40– 50	400	1200	1600
7	50– 60	"	1600	2000
8	60– 70	"	2000	2400
9	70– 80	"	"	"
10–16	80–120		2400	"

* Financial support was provided by Development Fund, Ohio State Univ., and Ciba.

Results. Never did any of the animals appear to suffer any adverse effects from the drugs. No signs of stiffness or joint involvement appeared. None of the animals exhibited a positive L-E prep., or positive serological reaction. Histologically, no abnormalities were found compatible with either rheumatoid arthritis or any other collagen disease. The adrenal cortex exhibited a histological change as reported previously(5), but is not pertinent to this report. Inspection of the animals at time of sacrifice by the Veterinary Service of the University revealed no gross abnormalities to distinguish our experimental animals from others of comparable age and weight that were being slaughtered at the same time.

Discussion. This experiment may be criticized for its shortness. However, spontaneous occurrence of arthritis in pigs commonly occurs when treatment of our animals was stopped. Further, we may assume the amounts and method of administering the drug should have resulted in sufficient blood level to produce a toxic or triggering-effect if any was to be obtained. We suggest a species specificity for the Hydralazine Syndrome, since occurrence of this syndrome has only been reported in man.

Summary. Pigs were given Apresoline in massive dosages over a 4 month period. The attempt to produce the Hydralazine Syndrome in this species of animals was a failure. A suggestion is made that there may be a species specificity in the action of the drug.

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Stimulation of Growth by Partial Hepatectomy.* (24876)

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We have previously reported that growth of certain transplanted rat tumors is enhanced by partial hepatectomy as compared with growth of these tumors in sham-operated controls(1). Previously, 3 groups of investigators had shown that partial hepatectomy in one of a pair of parabiotic rats induced increased mitoses in the liver of the other, intact, parabiont(2,3,4). Because these observations indicated enhancement of certain growth processes in the presence of regenerating liver, the effect of the latter was investigated further. Studies were made of the effect of partial hepatectomy and liver regeneration on: (a) mitotic rate in cornea and skin; (b) compensatory hypertrophy of kidney following unilateral nephrectomy; (c) epiphyseal width in the hypophysectomized rat.

Material and methods. Partial hepatectomy was performed under ether anesthesia by the method of Higgins and Anderson whereby 67% of liver is removed(5). Male rats of Wistar strain, obtained from Barkbridge Farms, N. J., were used, except in the study of epiphyseal width. The latter experiments were performed on hypophysectomized female rats of Sprague Dawley strain, purchased from Hormone Assay Labs, Chicago. Hypophysectomy was performed at the age of 26 days, and the experiments 2 weeks later. *Corneal mitosis.* Partially hepatectomized and control rats were killed by exsanguination under ether anesthesia on fourth day after operation; all animals received an injection of colchicine (1 mg/kg body wt) 8 hours prior to death. Control rats were subjected to "sham operation" on day on which the experimental group was partially hepatectomized. The sham operation consisted of laparotomy under ether anesthesia; liver lobes were pushed out of abdomen through the incision, then returned to abdominal cavity. At autopsy, the eyes were fixed in Bouin-Hollande solution; after fixation the cornea was dissected out, embedded in paraffin, and sectioned serially at 6 μ. Sections were stained with Mallory's acid-hematoxylin, or with Masson's iron-hematoxylin. 12-14 sections on either side of the equator were used for mitosis counts. Randomization of microscopic fields was performed by use of random numbers of Kendall and Smith(6). To avoid counting mitoses twice, only alternate sections were used. The mitotic index was calculated by the formula $\frac{\text{mitotic nuclei}}{\text{quiescent nuclei}} \times 100$. The Rank test, according to Kruskal and Wallis(7) was applied to evaluate significance (p). *Compensatory hypertrophy of kidney.* The experimental group was subjected to partial hepatectomy; at the same time one kidney was removed. In controls, only unilateral nephrectomy was performed. Weight of extirpated kidney and body weight were recorded. Three weeks later all animals were killed by exsanguination under ether anesthesia and right kidneys were weighed. The difference between weights of the 2 kidneys is a measure of compensatory hypertrophy following unilateral nephrectomy. The significance of difference of mean increments in kidney weight between control group (nephrectomy only) and nephrectomized hepatectomized group was determined by Fisher-"Student" test. *Epiphyseal growth in hypophysectomized rats.* Female rats hypophysectomized on 26th day of life were subjected to partial hepatectomy 2 weeks after hypophysectomy; hypophysectomized controls were subjected to sham operation as described above. In the first 2 series postoperative mortality was so high that no definite conclusion could be drawn from the few survivors. Thereafter, both hepatectomized and sham-operated animals were given 2.5 mg cortisone acetate by subcutaneous injection daily

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TABLE I. Mitoses in Cornea following Partial Hepatectomy.

	No. of animals	Median mitotic index
Controls	7	.722
Hepatect.	11	2.080
		$P_{(H)} < .001$*

* Rank Test (Kruskal and Wallis).

beginning on day of operation. All animals were killed on 6th day after operation. The tibiae were fixed in Bouin-trichloracetic acid, and decalcified in a solution containing 5% trichloracetic acid and 10% formaldehyde. After embedding in paraffin, serial sections were made of the region of proximal epiphysis and were stained with hematoxylin eosin. Measurements of epiphyseal width were made on central sections, with use of eye-piece micrometer(8). For statistical evaluation, the p of difference between the 2 groups was determined by Fisher's "Student" method.

Results. Mitosis stimulation, cornea and skin (Pinna). Four days after partial hepatectomy, the mitotic index in the cornea was significantly higher than in cornea of sham-operated controls (Table I). No increase of mitotic activity was found in the skin (pinna). (Not tabulated).

Compensatory enlargement of kidney. In rats subjected simultaneously to unilateral nephrectomy and partial hepatectomy, compensatory enlargement of remaining kidney was greater than in controls, subjected only to unilateral nephrectomy. The difference was statistically significant (Table II).

Growth of the tibial epiphysis in hypophysectomized rats. Six days after partial hepatectomy there was a highly significant increase in epiphyseal width as compared with that in sham-operated controls (Table III). The possible growth inhibiting effect of the administered cortisone acetate we consider of minor importance because both hepatectomized and control rats received identical treatment.

Discussion. Experiments here reported show stimulation of growth of 3 different tissues following partial hepatectomy. These 3 tissues differ widely in their biological characteristics. Intensive and rapid growth induced in liver remnant following partial hepatectomy has long been known. Occurrence of a mitotic reaction in liver of a normal, intact parabiont, following partial hepatectomy in the other, first demonstrated that a growth stimulus was carried humorally following partial hepatectomy(2,3,4). These data could be interpreted as indicating presence of an organ specific growth-stimulating agent. Our previously reported findings of increased tumor growth in the presence of regenerating liver(1) and extension of this effect in the present report, to non-tumor tissues, indicate that the growth stimulus is not organ specific.

The question whether such growth stimulation is due to an active growth stimulating agent circulating in partially hepatectomized

TABLE III. Effect of Partial Hepatectomy on Epiphyseal Width of Proximal Tibia of Hypophysectomized Rats.

	No. of animals	Mean epiphyseal width
Sham operated controls	17	212.0 ± 4.67*
Hepatectomized	10	246.7 ± 6.74
		$P_{(t)} < .001$†

* Stand. error of mean.
† T-test (Fisher's Student method).

TABLE II. Effect of Partial Hepatectomy on Compensatory Hypertrophy of Kidney.

	No. of animals	Mean incr. in kidney wt (g) Absolute	Relative*	Body wt incr. (g)
Control nephrect.	18	.320 ± .03†		57.9
Nephrect., hepatect.	8	.430 ± .04		50.5
$P_{(t)}$ of diff.‡		.05		
Control nephrect.	9	.321 ± .02	.080 ± .01	40 ± 14.2
Nephrect., hepatect.	7	.544 ± .07	.228 ± .05	55.4 ± 28.2
$P_{(t)}$ of diff.		<.01	<.01	<.2

* Wt of each kidney recorded in % of body wt at time kidney was obtained.
† Stand. error of mean.
‡ T-test (Fisher's "Student" method).

rats, possibly secreted by the regenerating liver, or whether it is due to removal of a growth inhibitor by partial hepatectomy, was first posed by Bucher *et al.*(2). Glinos has recently reviewed the evidence for the latter mechanism, implicating plasma proteins as the regulating factor, or the agent of "chemical communication"(9). On the other hand, Friedrich-Freksa and Zaki have found that injection of serum of hepatectomized, but not of intact rats into normal recipients induces mitoses in the liver. This is interpreted as evidence for presence of a growth-stimulating factor(10). Glinos discounts this report because he was unable to confirm it; however Stich and Florian have confirmed, in part, the findings of Friedrich-Freksa, inasmuch as they found that injection of serum from partially hepatectomized rats increased the mitotic count of regenerating liver in the recipient animal. Injection of serum from intact donors, or of homogenates of normal liver, inhibited mitosis in the regenerating liver. The authors also interpret their results as indicating regulation of liver growth by an inhibitor present in normal liver, and evidently in circulating blood(11). However the fact that serum from hepatectomized donors showed not merely absence of inhibiting action, but actual stimulation, suggests the possibility of a balance between stimulating and inhibiting factors(12). The studies cited above were concerned with regulation of liver regeneration and were interpreted in terms of organ-specific growth regulators. It is not known whether the non-organ-specific growth stimulation incident to liver regeneration here described, is subject to similar inhibitory and regulatory factors; this possibility is currently being investigated in our laboratories.

Summary. It has been shown by others that partial hepatectomy in one partner of parabiotic rats induced mitoses in liver of the other, intact parabiont. We found previously that growth of transplanted tumors was enhanced by partial hepatectomy of the host. In this paper stimulation of non-malignant growth following partial hepatectomy in the rat is reported. Mitotic rate in cornea, and compensatory hypertrophy of kidney is increased. In hypophysectomized rats partial hepatectomy is followed by increased width of tibial epiphysis. These findings are discussed with regard to organ specificity and mechanisms of growth regulation.

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Reversal by Metabolic Regulators of CO_2-Induced Inhibition of Mammalian Spermatozoa.* (24877)

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The causes of quiescence of mammalian spermatozoa in the epididymis and of their acquisition of active motility upon ejaculation have long been sought by physiologists. Lardy and his co-workers(5) found a metabolic regulator in epididymal spermatozoa of the bull, which diffused from the cells to stimulate them to markedly greater respiration. Bishop and Salisbury(2) after studying aerobic metabolic aspects of the "dilution effect" with ejaculated bull spermatozoa, had postulated a metabolic inhibitor in semen "associated with spermatozoa." They based their speculation on the fact that dilution of a standard number of cells with either seminal plasma or with 0.9% NaCl resulted in markedly increased rate of oxygen consumption/cell. At first inspection these observations, one dealing with a stimulator, the other with inhibitor, seem contradictory and no attempt has been made to reconcile them. However, recent studies indicate these observations to be part of the physiological change associated with maturation and production of fully active spermatozoa. Earlier identified as elemental sulfur by yeast-test then employed, the regulator contained that element(5) but its effects on respiration were duplicable only by cysteine, homocysteine, glutathione and inorganic sulfide and sulfite, the sulfite isolated as one of the important reducing substances in semen by Larson and Salisbury(6). Roedel in Germany verified the observations of the Wisconsin group and showed that epididymal spermatozoa contained a cysteine desulfhydrase which released sulfide from cysteine(8). The sulfide was effective in producing the same stimulation to respiration by epididymal spermatozoa as reported for diffusible substance containing sulfur. Sodium and chloride ions tend to stimulate metabolic activity of spermatozoa(2,9). K-ion at levels present in the epididymis inhibits respiration(4) and anaerobic glycolysis by ejaculated bull spermatozoa(12). Calcium ion interacts to counter the potassium effect(4). The p CO_2 above about 2% of atmosphere independently inhibits glycolysis(12), and at 100% CO_2 glycolysis is less than 10% of that occurring under N_2(11). Inhibition is reversible by replacement of CO_2 by air or by N_2(11). Evidence exists that epididymal sperm or ejaculated cells in a highly concentrated condition are low in chloride and high in CO_2(3,7) and on dilution a chloride shift occurs allowing CO_2 to be released from cells and chloride to enter them(7). Recent work reported here shows that sulfide or sulfite by-product of cysteine desulfhydrase activity by spermatozoa is fully capable of reversing inhibition of anaerobic glycolysis by CO_2. These suggest that diffusible substance in epididymal spermatozoa observed by Lardy *et al.*(5) and others(8) was probably not only a sulfhydryl decomposition product but possibly, in addition, CO_2, and that sulfide and sulfite from sulfhydryl component are catalysts of, if not active participants in, the reaction releasing CO_2 from the cell.

Procedure. The technic used has been described(12). Briefly it consisted of measurement of CO_2 evolution by acid metabolites from bicarbonate, or of lactic acid output, by ejaculated spermatozoa suspended in their own seminal plasma, and in IVT_{Na} diluent at 37°C for 4 hours under 3 p CO_2, of 5 and 50% in N_2 and 100% CO_2. The data, each a mean of 3 replicates, are reported as equivalent μg of lactic acid produced by 10^8 cells/hour during 4-hour incubation at 37°C. In 5 experiments the μg acid equivalent was calculated from μl CO_2 evolved from bicarbonate after correction for CO_2 and acid retention (12). In Exp. No. 3 in which levels of 0, 30, 300, and 600 μg of sulfur were added/ml diluent as glutathione, actual lactic acid determinations were made before and after the 4-hour period by method of Barker and Summerson(1). Motility of cells was determined

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TABLE I. Effect on Anaerobic Glycolysis and Livability of Bovine Spermatozoa during 4 Hr at 37°C of Various Amounts of Cysteine, Glutathione, Sodium Sulfide and Sodium Sulfite.

		Cysteine Exp. 1				Glutathione Exp. 2				Glutathione Exp. 3			
	pCO_2	μg S/ml				μg S/ml				μg S/ml			
	(%)	0	30	60	90	0	30	60	90	0	30	300	600
Z_{LA}*	5	148	141	132	133	156	155	150	144	82	90	67	70
	50	38	36	29	43	11	14	14	78	22	23	24	26
	100	15	14	11	15	3	9	10	9	13	10	10	18
Final mot., %	5	52	50	55	58	67	70	67	70	38	58	60	65
	50	57	60	57	48	80	73	73	70	52	35	63	65
	100	47	57	52	57	73	70	60	70	58	55	58	57

		Sulfide Exp. 4				Sulfide Exp. 5				Sulfite Exp. 6			
	pCO_2	μg S/ml				μg S/ml				μg S/ml			
	(%)	0	5	20	80	0	160	320	480	0	30	60	90
Z_{LA}*	5	168	158	155	161	142	116	174	173	130	171	160	153
	50	28	23	18	25	18	37	110	121	17	89	78	77
	100	10	12	14	18	3	45	105	129	6	94	89	85
Final mot., %	5	53	57	53	50	63	47	40	40	67	65	63	58
	50	53	57	63	63	60	53	20	30	63	53	52	60
	100	50	53	50	60	67	47	24	47	65	60	58	53

* Z_{LA} = μg lactic acid produced by 10^8 cells/hr, directly determined in Exp. 3, and calculated from CO_2 evolution from bicarbonate in the 5 other experiments.

at end of 4-hour incubation by direct microscopic examination after aeration of cells following their removal from Warburg flasks. Dilution rate was one part of semen to 4 parts of IVT_{Na} diluent. The buffer of this diluent was composed of sodium citrate and sodium bicarbonate (0.025M). Various quantities of cysteine and glutathione, as representative of sulfhydryl components in the epididymis, and of sodium sulfide, sulfite and sulfate, as representative of their breakdown products after desulfhydrase action(8), were added to the IVT semen diluent.

Results. The results of 6 experiments (Table I) clearly show the previously reported statistically and biologically highly significant inhibition of glycolysis by increasing p CO_2 in the gas phase surrounding the spermatozoa. Neither cysteine nor glutathione at levels employed, which were higher in the case of glutathione than the total non-carbohydrate reducing substances found in semen(10), had any measurable influence on glycolysis of ejaculated spermatozoa. There are, of course, variations in mean glycolysis values but, except for the effect of CO_2, none of the treatments approached statistical significance. In Exp. 3, however, ability of cells to withstand incubation as indicated by final motilities was improved by the glutathione.

Both sulfide and sulfite, on the other hand, each exerted a marked influence on inhibition of glycolysis by CO_2. The effect was to release inhibition by CO_2. In 100% CO_2, for example, without added sulfide, glycolysis was less than 3% of that occurring under 5% CO_2. However, in the presence of 480 μg S/ml as sulfide, glycolysis was approximately 75% as great as with only 5% CO_2. This level of sulfide was several times higher in sulfur equivalent than the levels reported in semen. However, sulfite at physiological levels(6) stimulated anaerobic glycolysis in 5% CO_2 and partially, though not completely, reversed inhibition by 50 and 100% CO_2. Additional sulfite over minimum level of an equivalent of 30 μg S/ml did not increase the response. All levels of sulfide in Exp. 5 depressed final motility.

A final experiment was replicated 3 times with 0, 30, 60 and 90 μg of S/ml as sodium sulfate added to diluent. It resulted in no measurable effect of the sulfate ion on motility nor on glycolysis, measured as acid equivalents of CO_2 evolved from bicarbonate, as hexose uptake, or as lactic acid produced.

These results and earlier observations lead to the speculation that phenomena of this sort are involved in initiation of motility of spermatozoa at ejaculation. Spermatozoa are inhibited by high p CO_2 and high K concentration in the epididymis. The enzyme or enzymes of spermatozoa responsible for cleavage of sulfide from the substrate, probably also from the cells, are probably activated by the citrate added to cells on admixture of accessory gland fluids at ejaculation. After activation, the sulfide diffuses from the cells to be converted to sulfite. The CO_2-release stimulated by these compounds, along with dilution of K by sodium chloride and uptake of hexose, result in reversal of the metabolic inhibition found in the epididymis.

Summary. Neither cysteine, glutathione nor sulfate exhibit a measurable effect on anaerobic glycolysis nor on its inhibition by high p CO_2. Sulfide, at higher than physiological levels, and sulfite, in amounts found in semen, stimulate anaerobic glycolysis and reverse inhibition of glycolysis by CO_2.

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Effect of Plasma from Wounded Donors on Nitrogen and Sulfur Excretion.* (24878)

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Several laboratories have presented data which seem to indicate that injured cells produce or cause production of a factor or factors having ability to alter rate of healing of wounds. These data have been obtained by observing the effects of extracts of crushed tissues when applied directly to healing wounds or when administered parenterally to wounded animals(1-3). Sandblom(4) and Auerbach(5) reported that the presence of injured tissue in the body causes production of factors appearing in the circulation, which affect rate of healing. They also observed that, when 2 wounds are consecutively inflicted in the same animal, rate of healing of the second wound appears affected by presence of the previously inflicted wound. Metabolism of nitrogen and sulfur have been shown to be markedly altered during healing of experimental wounds. As a result of wounding, the amount of urinary nitrogen increases sharply, while at the same time there is a relative retention of sulfur compounds by the wounded animal(6-9). At this time also, proteins rich in cystine and methionine are synthesized in regenerating wound tissue; these newly synthesized proteins must be considered to be distinct from collagen, since this latter protein contains no cystine and very little, if any, methionine(14). In this study, the effects of factor(s) released into blood by the stimulus of wounding, as they affect me-

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tabolism of nitrogen and sulfur, were investigated.

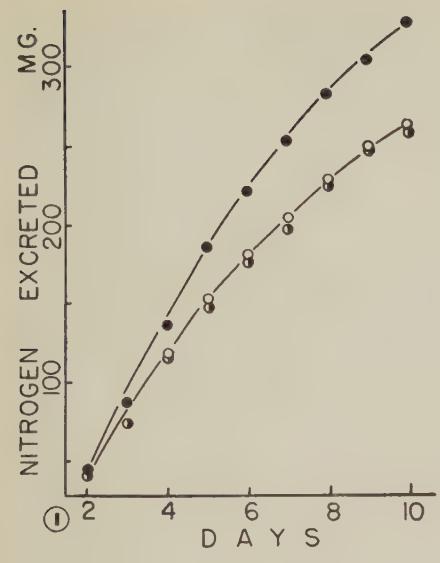
Methods. Adult female white rats weighing 220 ± 20 g were used. Wounded animals used as source of plasma, and animals for the metabolic studies were kept in individual metabolism cages during experiment. Rats used as blood donors were fed commercial stock diet, *ad lib.*, while those used in metabolic studies were given 8 g of protein-free diet daily(12). Each experimental rat completely consumed this diet in 24 hours. Plasma from donor rats was obtained as follows. After anesthetizing rats with nembutal, half of the animals were wounded by excising a circle of skin 4 cm in diameter, through the fascia to the muscle, in region of neck and shoulder blades. On day following wounding, and for 4 to 6 days thereafter, 2-4 ml samples of blood were obtained from each animal by heart puncture. Heparin was used to inhibit clotting. Plasma was immediately centrifuged free from cells and dried by lyophilization. The dried plasma was pooled and stored until used, in a desiccator at 5°C. Comparison of the effect of plasma from normal and wounded animals, on nitrogen and sulfur metabolism was undertaken by using 3 groups of 8 rats. These animals were acclimated to the protein-free diet for 3 days prior to start of experiment. Thereafter, each rat in Group I was given intraperitoneally 50 mg of dried plasma obtained from wounded rats (redissolved in 0.5 ml of water); animals in Group II were given the same amount of plasma from normal unwounded donors, while those in Group III were injected with 0.5 ml of 0.15 *M* NaCl solution. These solutions were administered twice daily at about 12 hour intervals. Each rat was also given an isotonic saline solution containing 5 x 10^6 counts/minute of S^{35} labeled L-cystine. Labeled amino acid was given only once at time of first administration of plasma. Administration of pooled plasma and saline were continued for 10 days. During experimental period, 24-hour urine samples were collected and analyzed for nitrogen, total sulfur and S^{35}. Nitrogen was determined by micro-kjeldahl method; sulfur by modification of the method of Toennies and Bakay(10), the digestion of samples being carried out with concentrated nitric acid and 30% hydrogen peroxide. S^{35} activity was determined by counting $BaSO_4$ samples at infinite thickness in Tracerlab SC-16 windowless gas-flow counter.

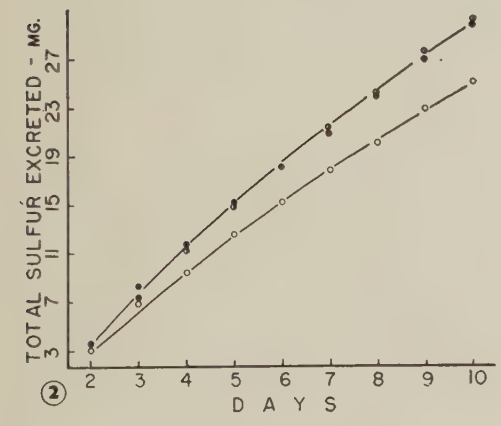
Results. Comparison of total urinary nitrogen excreted by rats in 3 experimental groups is plotted in Fig. 1. Animals receiving plasma from wounded donors and those given isotonic saline excreted the same amount of nitrogen. This level of excretion was significantly less than nitrogen output of rats given plasma from normal donors. Since plasma nitrogen administered to animals in Groups I and II was essentially the same, the difference in nitrogen excretion must be attributed to some cause other than nitrogen intake. Differences in nitrogen output are inexplicable unless one considers the possibility of qualitative differences in plasma obtained from wounded and normal donor animals. It then seems probable that these effects may be brought about by some substance of a hormonal nature.

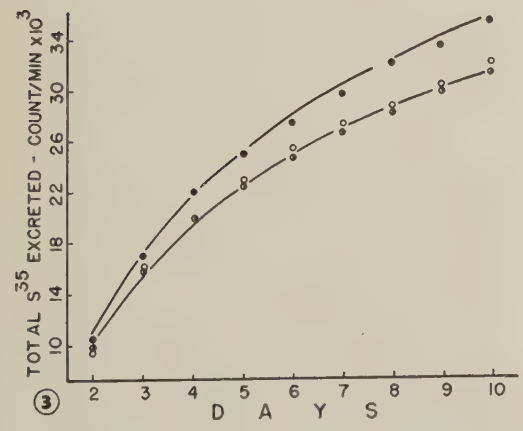
The data on excretion of sulfur (Fig. 2) indicate that significantly less was excreted by animals receiving plasma from wounded rats as compared to those receiving normal plasma. This situation is somewhat analogous to that observed when excretion of sulfur by wounded rats is compared to that in unwounded control rats(13).

In Fig. 3 is presented excretion of S^{35} by the 3 groups of rats. Again, significantly more of the isotope is excreted by rats given normal plasma than by the other 2 groups. Administration of plasma from normal rats seems to have stimulated an increased excretion of S^{35}-labeled metabolites, as well as nitrogen, while plasma from wounded donors appears to cause retention of labeled sulfur. It is possible that differences in the metabolic picture displayed by rats in Groups II and III may be due to additional protein available to animals in Group II.

On 10th day of experiment, the rats were killed and weight of various organs among the 3 groups was compared. No significant difference in organs from each group was







Open circles, Group I, received plasma from wounded donors; *solid circles,* Group II, received normal plasma; *half-solid circles,* Group III, received isotonic saline.

TABLE I. Organ Weights of Rats Given Plasma from Normal and Wounded Rats.

Group	Kidney†		Liver†	
	Organ wt, g	g/100 g body wt	Organ wt, g	g/100 g body wt
I *	1.577	.828	6.01	3.20
II	1.860	1.000	8.64	4.58
III	1.451	.797	6.50	3.57

* Group I, 100 mg of plasma solids/rat/day from wounded donors. Group II, 100 mg of plasma solids/rat/day from normal donors. Group III, 1.0 ml of saline/rat/day.

† Statistical difference between Group I and II and between II and III: $p < 0.01$. No significant difference between Groups I and III.

found except in the case of liver and kidneys, which were heavier in animals of Group II (normal plasma) than in the other groups (Table I). It seems probable that the greater weight of these organs, as well as the greater excretion of nitrogen and sulfur by rats receiving normal plasma reflects some degree of loss of muscle protein. The possibility that stress of repeated withdrawals of blood may be sufficient to cause increased plasma ACTH level(11) cannot be discounted. Thus, it may be considered that administration of normal plasma resulted in changes in metabolism of protein deficient animals, and that these changes were counteracted by some substance in plasma obtained from wounded animals.

Summary. Rats, on a protein deficient diet, were given reconstituted plasma from wounded donors. These animals excreted less nitrogen and sulfur than did comparable rats given plasma from normal donors. Similar results were obtained on excretion of S^{35}, administered as labeled cystine. After 10 days, liver and kidney of rats receiving normal plasma were significantly heavier than in rats getting plasma from wounded animals. The data appear to indicate that plasma from wounded rats contains a factor(s) which affects metabolism of nitrogen and sulfur.

FIG. 1. Total cumulative output of nitrogen in mg/day.

FIG. 2. Total cumulative excretion of sulfur in mg/day.

FIG. 3. Total cumulative excretion of S^{35} in counts/min. $\times 10^3$, corrected for decay to time of administration. S^{35} was administered in form of cystine-S^{35}.

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Bovine Ocular Squamous Cell Carcinoma II. Tissue Culture Studies of Papilloma.* (24879)

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Studies on pathological anatomy of bovine ocular squamous cell carcinoma demonstrated eosinophilic, cytoplasmic inclusion-like bodies in cells of both benign precursor and malignant lesions(1). Plaque and papilloma, benign precursor lesions, exhibited bi-laterality, disappearance and reappearance, which suggested a virus may be involved in the etiology of this disease. Tissue culture studies of cells derived from plaque lesions(2) have shown characteristic changes suggestive of a filterable agent. As a result of these preliminary findings, tissue culture studies of papilloma were undertaken to determine whether similar changes also occur in cells from papilloma grown *in vitro.*

Materials and methods. The method of removal of the lesions, treatment for transportation to the laboratory, subsequent treatment in laboratory and methods used for detailed study of cellular outgrowths from lesions have already been described(2,3,4).

Results. Phase contrast microscopy of cells derived from papillomata growing in specially designed chambers(2) has confirmed and extended preliminary observations on stained cultures in T-30 culture flasks examined at low magnifications by bright field microscopy. The cells derived from papillomata are epithelioid in character, relatively uniform in size measuring 0.01 mm in diameter, and frequently multinucleated (Fig. 1). Occasional large cells are seen as much as 0.03 mm in diameter; these cells are always multinucleated. The cells exhibit well marked tonofibrils, mitochondria long and interlacing are well developed (Fig. 2). Pinocytosis is marked, many cells showing cytoplasmic fimbriae (Fig. 3). Cells frequently exhibit vacuolisation of cytoplasm. Such cells occur in scattered foci. Vacuoles are found in the perinuclear zone and contain dense homogeneous material (Fig. 4). Cytoplasmic inclusions, irregular in outline, and not associated with presence of vacuoles also occur (Fig. 5). The nuclei contain multiple nucleoli of variable size and shape, and occasionally show margination of chromatin (Fig. 6). As in cells derived from plaque lesions, vacuolisa-

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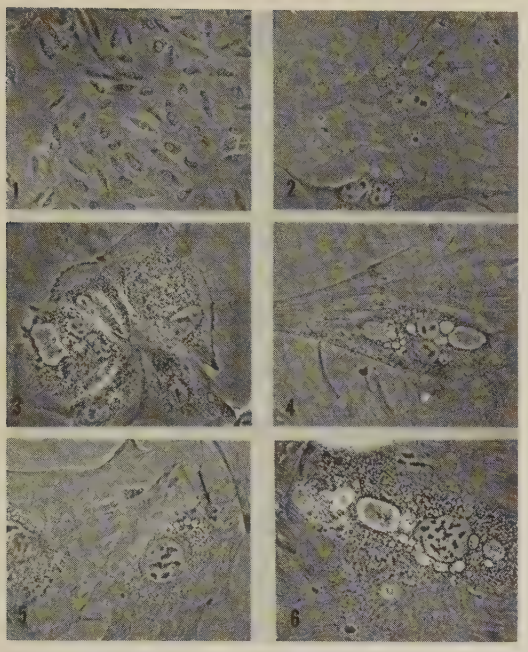

Photographs were made with the phase optics on a Zeiss Microscope, Model No. GFL-65, using the 35 mm camera attachment.

FIG. 1. Cells derived from papilloma, a precursor lesion of bovine ocular squamous-cell carcinoma. 22nd passage, 11 day S-M chamber culture. × 70.

FIG. 2. Cell showing well developed mitochondria and cytoplasmic inclusion. 5th passage, 3 day S-M chamber culture. × 195.

FIG. 3. Cells showing well developed cytoplasmic fimbriae. 22nd passage, 8 day S-M chamber culture. × 350.

FIG. 4. Part of a cell with perinuclear vacuolisation and early inclusion formation. 22nd passage, 11 day S-M chamber culture. × 350.

FIG. 5. Cells showing perinuclear vacuolisation and cytoplasmic inclusions. 12th passage, 7 day S-M chamber culture. × 350.

FIG. 6. Part of a large cell with nucleus showing multiple nucleoli and early margination of chromatin. Perinuclear vacuoles containing early inclusions are also seen. 22nd passage, 9 day S-M chamber culture. × 350.

tion of cytoplasm, when it occurs, spreads peripherally to adjacent cells and leads to loss of culture. Vacuolisation is less frequent and occurs later in cell cultures derived from papillomata than in cultures derived from plaque lesions. It has been observed in cells of cultures of seventh and subsequent passages. Cultures of a number of papillomata have been maintained for more than 50 sequential passages over 30 weeks. Thirty-three papillomata have been put into culture: 4 were maintained for 4 weeks or less; 17 for more than 8 weeks; 9 for more than 18 weeks and 3 for more than 30 weeks. Cells derived from 7 papillomata are presently in culture, 3 have been in culture for more than 30 weeks, 4 for more than 12 weeks. Studies are in progress to determine the nature of changes which lead to loss of cultures.

Summary. Properties of cells derived from papillomata lesions of bovine ocular squamous-cell carcinoma grown *in vitro* are described. The cells have been observed, after 7 or more passages, to exhibit cytoplasmic changes which commonly result in loss of the culture. In some cultures changes have not been observed after more than 50 passages. The nature of the changes is being investigated.

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Effect of Estrogen upon Thyroxine Secretion Rate in Intact Female Rats.* (24880)

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Estrogen administration has been reported to increase(1-5), to have little or no effect(6-8), and to depress thyroid activity(4,9). Some investigators have indicated that estrogens may stimulate or depress thyroid function, depending upon dose and period of treatment(1,4,10,11). In these studies many indices of thyroid function have been used, such as I^{131} uptake, rate of thyroidal I^{131} release, hyperplasia and thyroid cytology. In view of conflicting results and limitations of some methods used in assessing thyroid activity, it seemed desirable to reinvestigate the influence of various levels of estrogen upon thyroid activity in female rats. This was done by employing the technic of thyroxine replacement and thereby estimating daily thyroxine secretion rate.

Materials and methods. Young mature female rats of Sprague-Dawley-Rolfsmeyer strain weighing 240-260 g were housed for 3-4 weeks after purchase, under uniform temperature (78-80° F) in animal room artificially illuminated during regular daylight hours. They were given Purina Lab Chow and fresh water daily. *Exp. I.* Seventy-one rats were injected intraperitoneally with 2 μc of carrier-free I^{131}. Forty-eight hours were allowed for fixation of I^{131} by thyroid gland. External neck counts were made at this time and at 2-day intervals thereafter by first anaesthetizing each animal with ether, then placing it on lead plate with thyroid region over a scintillation probe containing a 1″ NaI crystal. Measurements of thyroidal radioactivity were made with a scintillation counter, Nuclear-Chicago (N.C. Model DS5-3) connected to rate meter (N.C. Model 1620A). Conventional corrections were made for radioactive decay and background. After initial thyroid count, subsequent neck counts were made on days 4 and 6 after I^{131} injection to establish that thyroidal I^{131} release was proceeding normally. Fifty-seven rats were injected subcutaneously with 1, 3.6, 15 or 50 μg/day estradiol benzoate (E.B.) in .1 ml olive oil commencing 5 days after injection of I^{131} and continuing until thyroid secretion rate was reached (5-9 days). Fourteen control rats were similarly treated with .1 ml olive oil/day. Each rat was injected subcutaneously with .5 μg/100 g/day l-thyroxine for 2 days commencing 6 days after I^{131}. Dose was increased at .5 μg/100 g increments, each level for 2 consecutive days with thyroid count taken the day of each increase. Thyroxine secretion rate (TSR) was estimated by plotting percentage of previous count with thyroxine dose. The dose which prevented further thyroidal I^{131} output in each rat (95-100% of previous count) was estimated as its TSR. *Exp. II.* Fifty-eight additional female rats were injected with 1, 3.6, 15 or 50 μg/day E.B. for 5 days. Eighteen served as controls of which half were injected with .1 ml olive oil/day. Body weight of each rat was recorded daily. Twenty-four hours after last injection, each animal was weighed and killed with ether. Thyroid, pituitary and adrenal glands were rapidly removed, blotted to remove surface moisture and weighed to nearest .1 mg on a Roller-Smith Torsion balance.

Results. The influence of various levels of E.B. upon average daily thyroxine secretion rate (TSR) of young mature female rats is presented in Table I. Only 3.6 μg/day resulted in significant increase of 35.5% over control value of 1.24 μg/100 g/day l-thyroxine. Body weight was not adversely affected by injection of 1 or 3.6 μg/day E.B. for 5 days. Administration of 15 or 50 μg/day for same period, however, caused a decrease in body weight of 2 and 5.7% respectively (Table II). Each rat was given the same quantity of food each day but those injected with

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TABLE I. Effect of Estradiol Benzoate upon Thyroxine Secretion Rate in Female Rats.

EB treatment (μg/day)	No. of rats	Initial body wt (g)	Thyroxine secretion rate (μg/100 g/day l-thyroxine)
Control	14	244.4	1.24 ± .10
1.0	15	253.9	1.13 ± .11
3.6	14	244.3	1.68 ± .06*
15.0	14	253.5	1.29 ± .09
50.0	14	256.0	1.25 ± .07

* Significant at 1% level.

15 or 50 μg/day apparently did not consume as much food as control animals since food progressively accumulated in their cages during period of treatment. Because of loss in body weight resulting from treatment with larger doses of E.B., weights of pituitary, thyroid and adrenal glands are expressed as mg/100 g initial body weight (Table II). Injection of 1 or 3.6 μg/day did not affect thyroid weight but significant increases of 18.6 and 26.7% occurred following administration of 15 and 50 μg/day for 5 days. Adrenal weights were lower in all groups with a significant depression occurring only in the 50 μg/day group. Pituitary weight, however, was significantly increased over controls in all but the 1 μg/day group. Maximum pituitary weight increase (42.6%) resulted from treatment with 15 μg/day level of E.B.

Discussion. The effect of various estrogens upon thyroid function has interested many investigators. Results however, have been conflicting due in part to varying time-dose relationships, species or strain differences and in technics used in evaluating thyroid function. Since I^{131} became available, more sensitive methods have replaced those based on morphological changes of thyroid gland. The 2 most widely used, I^{131} uptake and rate of release of thyroidal I^{131}, are, however, qualitative, and values obtained cannot be translated into quantitative terms(12). The technic of measuring daily thyroxine secretion rate (TSR) is a quantitative measure of thyroid activity based upon the classical procedure in physiology of hormonal replacement. Results of our study based on this method clearly indicate that a relatively low level of estradiol benzoate (3.6 μg/day) administered for a short period markedly increased daily thyroid hormone output, whereas higher levels had no effect. Increase in TSR was not, however, associated with increase in weight of thyroid gland. It has been reported that estrogen depresses appetite and body weight in laboratory animals(13,14) which may lead to diminished thyrotropin secretion by the pituitary gland(5,13). The failure of 15 and 50 μg/day E.B. in our study to increase significantly TSR, may perhaps be explained on this basis since a decrease in appetite and body weight occurred. These levels, however, elicited marked increase in thyroid weight which may indicate a compensatory enlargement has occurred as a result of partial inanition(5). It is also possible that high levels of E.B. stimulate the thyroid directly(1) or that thyroid weight increase may be due in part to the well known vasodilating action of estrogen with resulting hyperemia.

It is thought that the increasing level of estrogen secreted during late pregnancy is responsible for increased secretion of lactogenic hormone by the pituitary(15). The significant increase in TSR obtained in our study with a dose of E.B. (3.6 μg/day), slightly higher than the accepted physiological dose in rats, suggests that increased secretion of estrogen in late pregnancy may also provide the stimulus for secretion of increased amounts of thyrotropin and, subsequently, more thy-

TABLE II. Some Effects of Estradiol Benzoate in Female Rats. (E.B. inj. daily for 5 days.)

EB treatment (μg/day)	No. of rats	Body wt (g) Initial	Final	% change	Avg Pituitary	Adrenals	Thyroid
					mg/100 g initial body wt		
Control	18	254.3	257.7	+1.3	4.79 ± .15	27.3 ± .81	4.68 ± .15
1.0	15	256.2	258.6	+ .9	5.08 ± .13	25.7 ± .63	4.50 ± .21
3.6	14	246.4	248.0	+1.1	5.83 ± .27*	25.3 ±1.16	4.61 ± .16
15.0	15	250.4	245.4	−2.0	6.83 ± .22*	26.6 ±1.09	5.55 ± .26*
50.0	14	253.1	238.7	−5.7	6.55 ± .26*	24.5 ± .98†	5.93 ± .30*

* Significant at 1% level from control. † Significant at 5% level from control.

roxine for lactation. This would provide an explanation for the significant increase in thyroid hormone output which occurs during early lactation in rats(16) and sheep(17).

Summary. The effect of various levels of estradiol benzoate (E.B.) upon thyroxine secretion rate (TSR) has been studied in young mature female rats. A dose of 3.6 μg/day E.B. caused significant increase of 35.5% in TSR over average control value of 1.24 μg/100 g/day l-thyroxine while 1, 15 or 50 μg/day had no effect. In another experiment, body weight and thyroid weight/100 g were not affected by injecting 1 or 3.6 μg/day E.B. for 5 days. Thyroid weight/100 g was significantly increased while appetite and body weight were markedly reduced following 15 or 50 μg/day for same period. Pituitary weight/100 g was significantly increased in all but the 1 μg/day group. Adrenal weights/100 g were lower in all groups with significant depression occurring in 50 μg/day group. These data are discussed in relation to stimulation or depression of thyroid activity by estrogen.

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Effect of 2-PAM on Neuromuscular Blockade Induced by Certain Chemicals. (24881)

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Holmes and Robins(1) found that 2-formyl-1-methylpyridinium iodide oxime (2-PAM) induces paralysis of isolated phrenic nerve-diaphragm preparation of the rat; the curaremimetic action of this compound has been corroborated for calf muscles of the cat, excited *in situ* through sciatic nerve stimulation by ourselves and for adductor muscle of human little finger, excited by stimulation of ulnar nerve(2). In addition, 2-PAM (5×10^{-3} M) decreases rate of shortening of isolated rectus abdominis of the frog in response to acetylcholine, but smaller concentrations of 2-PAM increase this rate(3). Grob and Johns reported also(2) that some patients given 2-PAM intravenously experienced thereafter orthostatic tachycardia and hypotension. These findings, and those that 2-PAM is particularly active in promoting reactivation of inhibited cholinesterase in cholinergic neurons of ganglia(4) and in motor end-plates of skeletal muscle(4,5), suggest that the oxime has a special affinity for nicotinic effectors, where it has a depolarising action of its own and also competes with other quaternary molecules (acetylcholine, for example) for the receptor site. Wagley(6) found, indeed, a reversible increase of about 40% in end-plate potential of frog muscle treated with 2-PAM *in vitro;* this is the type of change expected from a de-

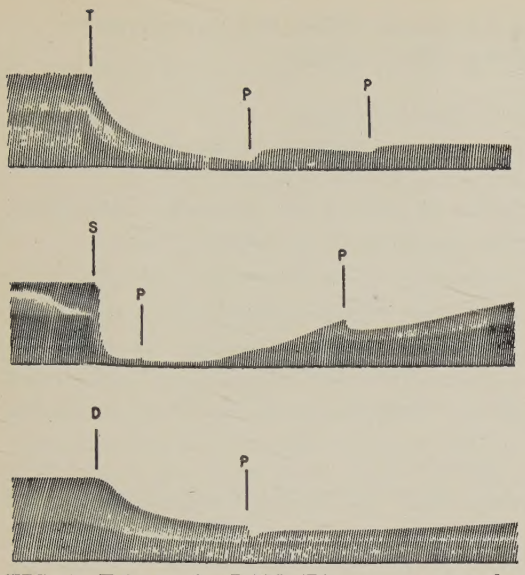

FIG. 1. Effects of 2-PAM (P) on neuromuscular block induced in the cat by d-tubocurarine (T), succinylcholine (S) or decamethonium (D).

polarising agent. Contradictory of the above suggestion is the report of Holmes and Robins (1) that oximes had no effect on transmission blocks induced in isolated phrenic nerve-diaphragm preparation by d-tubocurarine, succinylcholine or decamethonium.

Methods. The present work was undertaken to restudy effects of 2-PAM on acute neuromuscular block. Cats prepared in our usual manner(7) were used, contractions of the gastrocnemius-soleus-tibialis anticus muscle group in response to electrical stimulation (one shock every 2 seconds) of the sciatic nerve being recorded kymographically. Intravenous injections of d-tubocurarine chloride(0.1 mg/kg), of succinylcholine chloride (0.05 mg/kg) or of decamethonium bromide (0.06 mg/kg) were used to produce a marked decrease in response of muscle to just supramaximal nerve stimulation. During period of reduced response, one or 2 intravenous injections of 5 mg/kg of 2-PAM were made.

Typical *results* are shown in the Figure. Injection of 2-PAM into cats treated previously with d-tubocurarine induced some increase in response to motor nerve excitation; similar injections into cats given initially succinylcholine or decamethonium intensified the decrement of response induced by the first compound.

These results, unlike those of Holmes and Robins(1), show that 2-PAM has effects on neuromuscular blocks induced by d-tubocurarine, succinylcholine or decamethonium. That block induced by d-tubocurarine is antagonised by 2-PAM but that those induced by succinylcholine or decamethonium are intensified by oxime supports the idea that 2-PAM has a depolarising action at the neuromuscular junction, antagonising block produced there by competitive blocker d-tubocurarine and intensifying those produced by depolarising blockers succinylcholine and decamethonium.

Summary. In the anesthetized cat, i.v. injection of 2-PAM lessens neuromuscular block induced by d-tubocurarine but intensifies that induced by succinylcholine or decamethonium. In addition to being a reactivator of cholinesterase inhibited by P-containing anticholinesterases, 2-PAM may depolarise the neuromuscular junction.

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Influence of Compound 48/80, Protamine and Other Substances on Rabbit Blood Basophils *in vitro*.* (24882)

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Basophil leucocytes and tissue mast cells have some morphological similarities and have been claimed to be functionally related(3,6,7). Both cells have been reported to contain histamine(8,13), heparin or heparin-like substances(9,10) as well as hyaluronic acid(1,4). In certain species, tissue mast cells contain serotonin (5-hydroxytryptamine)(5). Asboe-Hansen and Glick(2) showed that, *in vitro*, the histamine liberator substance 48/80 and protamine sulfate prevented metachromatic staining of rat peritoneal mast cells. These agents and serotonin caused clumping of the cells. Hyaluronidase partially removed the granules of mast cells. *In vivo*, compound 48/80 brings about a decrease in blood basophil count in rabbits(6). The present work was undertaken to study the influence of substances related to mast cell physiology on blood basophils *in vitro*.

Methods. Rabbits with high basophil counts were chosen. Blood obtained from ear vein and collected in tube containing dried anticoagulant mixture of ammonium and potassium oxalate was used. Fifty μl of blood was added to 50 μl of the agent dissolved in sterile Tyrode solution at pH 7.4. After mixing, blood agent mixtures were incubated at 37°C for 20 minutes. Thereafter, a sample of blood agent mixture was diluted 1:5 with modified(6) Moore and James toluidine blue diluting fluid(11). To control the possible effect of plasma protein parallel experiments were performed using blood corpuscles washed 3 times with sterile Tyrode solution. Chamber counting of all white blood cells and of basophils was performed as previously described(6). In each sample 200 basophils or more were counted. Controls were carried out using similar volume of sterile Tyrode solution at pH 7.4 for incubation with blood. The agents used are listed in Table I.

Results. Control basophil and total white cell counts were considered 100%, and to this the other counts were related. There was no apparent difference between whole and washed blood. Both compound 48/80 and protamine sulfate caused a decrease in number of basophils but not in total white cell count. The same effect was observed whether incubation with either agent was performed before or after staining with toluidine blue. Histamine, serotonin and hyaluronic acid which are supposed to be contained in mast cells, did not influence the count of basophil leucocytes or of white cells. As seen in Table I, basophil and total white cell counts were lower in blood samples stained with toluidine blue after incubation with heparin. If stained with gentian violet acetic acid solution, the total white cell count did not differ from control count. Normal counts were also observed after incubation with histamine antagonists mepyramine and pyribenzamine, the serotonin releaser reserpine, and with testicular hyaluronidase.

Discussion. In some respects, the results of this experiment accord with that performed by Asboe-Hansen and Glick(2) on isolated rat peritoneal mast cells. The influence of histamine releaser compound 48/80 and of protamine on basophils, was like that on mast cells, destaining and preventing metachromatic staining of their granules. Both substances are known to have a certain affinity for acid mucopolysaccharides(12). Hyaluronidase did not break down the granular content. Heparin, an acid mucopolysaccharide with strong affinity for metachromatic stains, when added to blood sample, appeared to bind most of the toluidine blue. Consequently, the rest of the dye was unable to render all basophils or other leucocytes detectable. A control experiment involving gentian

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TABLE I. *In Vitro* Influence of Various Agents on Basophil and Total White Blood Cell Count in Rabbit Blood.

Agent	Final conc., %	Counts in % of control: Basophils	Total leucocytes
A. Incubation before toluidine blue staining:			
Heparin* (110 i.e./mg)	.5	76	39
Histamine hydrochloride	"	102	104
Serotonin creatinine sulphate†	"	113	90
Hyaluronic acid‡ (umbilical cord)	"	100	80
Protamine sulphate§	"	15	107
Mepyramine	"	99	89
Pyribenzamine‖	"	102	101
Compound 48/80¶	"	8	115
Reserpine	"	99	110
Testicular hyaluronidase‡ (100 VRU/mg)	1.0	97	103
Idem‡ (3000 ")	.1	109	104
" (heated at 70°C, 40 hr)	"	86	99
B. Incubation before gentian violet staining:			
Heparin*	.5		104
C. Incubation after toluidine blue staining:			
Protamine sulphate§	.5	43	105
Compound 48/80¶	"	0	90

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violet staining substantiated this view.

Summary. Metachromatic stainability of rabbit basophils was prevented by compound 48/80 and protamine. If already stained with toluidine blue, the stain was abolished. Heparin apparently combined with the toluidine blue stain, thus accounting for a decrease in counts of basophils and other white cells. Hyaluronic acid, histamine, serotonin, as well as testicular hyaluronidase, antihistamines mepyramine and pyribenzamine, serotonin releaser reserpine, produced no change in count of basophil leucocytes or other white blood cells.

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